DO NOT CIRCULATE

UNIVERSITY OF MICHIGAN
NOV 16 1953
MEDICAL LIBRARY

VOL. XXXIV No. 5] [OCTOBER, 1953

THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY

EDITOR
L. P. Garrod

ASSISTANT EDITORS
C. F. Barwell, G. J. Cunningham, H. Lehmann, R. A. Shooter

ADVISORY EDITORS
M. P. Curwen, J. R. Marrack, A. A. Miles, W. T. J. Morgan, C. G. Pope, R. W. Scarff, F. R. Selbie, Wilson Smith, D. D. Woods

with the support of

S. P. Bedson, E. C. Dodds, C. L. Evans, H. W. Florey, H. D. Kay, J. W. McLeod, W. J. Tulloch

DIRECTOR
Paul Fildes

LONDON
H. K. LEWIS & Co. Ltd.
136 GOWER ST., W.C.1

on behalf of

The British Journal of Experimental Pathology. Registered Office: 120 Moorgate, London, E.C.2

Annual Subscription, January to December, post free, Four Pounds net
Single Numbers, Sixteen Shillings net

PAGE
341
347
365
376
384
392
400
412
420
426
441
458
464

THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY.

EDITORIAL NOTICE TO CONTRIBUTORS.

Scope of Papers.—This Journal was founded for the publication of original communications describing the technique and results of experimental researches into the causation, diagnosis and cure of disease in man. Papers upon bacteriological, biochemical, pharmacological, physiological, serological and other subjects are therefore acceptable when bearing directly upon these objects.

Forwarding Papers and Correspondence.—Papers should be forwarded to Professor L. P. Garrod, Department of Pathology, St. Bartholomew's Hospital, London, E.C. 1.

General Correspondence relating to the conduct of the Journal should be addressed to Sir Paul Fildes, Sir William Dunn School of Pathology, University of Oxford.

Subscriptions and business correspondence are received by the publishers, Messrs. H. K. Lewis & Co. Ltd., 136, Gower Street, W.C. 1.

Date of Publication.—The Journal is published bi-monthly, in the middle of February, April, June, August, October, and December.

Rapidity of Publication.—Every endeavour is made to publish papers as soon as possible, but under present conditions a delay of 4–6 months between receipt and publication can rarely be avoided.

Proofs.——Proofs will in future be submitted to authors in any part of the world. Prompt return by air mail from distant contributors is necessary if publication is not to be delayed.

Reprints.—25 reprints will be supplied free to authors who are registered as personal subscribers to the Journal. Additional copies must be ordered from the Publishers *before the issue is printed.*

Estimates for reprints will be supplied on request to the Publishers.

Made and printed in Great Britain.

Vol. XXXIV, No. 4 (August, 1953), was issued on 16.ix.53.

THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY

VOL. XXXIV OCTOBER, 1953 NO. 5

A METHOD FOR COLLECTING BACTERIA AND THEIR PRODUCTS FROM INFECTIONS IN EXPERIMENTAL ANIMALS, WITH SPECIAL REFERENCE TO *BACILLUS ANTHRACIS*.

H. SMITH, J. KEPPIE AND J. L. STANLEY.

From the Microbiological Research Department, Porton, nr. Salisbury, Wilts.

Received for publication April 29, 1953.

THERE is abundant evidence that the complex metabolic activity of a pathogen in establishing a disease under the cultural conditions of the host's tissues is different from its metabolism *in vitro*. Thus, the change in morphology and colony appearance and the decrease in virulence which occurs on subcultivating recently isolated organisms is well known. The method of increasing the virulence of organisms by animal passage means that substances and processes connected with virulence are produced *in vivo* (possibly as a result of mutation), and not to any significant extent by culture *in vitro*. A similar conclusion can be drawn from the difficulty or impossibility of distinguishing between virulent and avirulent strains of certain species such as *Pasteurella pestis* (Wilson and Miles, 1948 ; Jawetz and Meyer, 1943 ; Pollitzer, 1952) by *in vitro* tests. Perhaps the most striking instances of the difference are those in which the use of living vaccines forms the only effective means of preventing the disease.

It seems, therefore, that chemical extraction of organisms and their products obtained from infected animals would be fruitful, particularly in connection with studies on virulence. Chemical fractionation of body fluids containing extracellular products of an infecting organism has been done before (*e.g.*, Cromartie, Watson, Bloom and Heckly, 1947). This is not so for the bacterial substance. Up to the present, organisms grown *in vivo* have not been obtained in large enough quantity and of sufficient purity for a chemical examination of their products and metabolism.

This paper describes a method for collecting *Bacillus anthracis* and its products from infected guinea-pigs for use in a study of virulence described in the next paper (Smith, Keppie and Stanley, 1953). The same method has been tried on a small scale for the separation of products from infections with several different species of pathogenic bacteria. It was equally successful as regards the collection of bacteria free from blood cells. Briefly, the method consists of collecting the blood and thoracic and peritoneal exudates from infected animals and removing the blood cells from the bacteria and their products by differential centrifugation

at low temperatures. The general problems in obtaining a sufficient quantity of materials and bacteria of adequate purity are as follows :

To ensure a sufficient quantity of bacteria, only those diseases which produce a large number of organisms in accessible locations of the host could be studied. It is inevitable that only a relatively small proportion of the organisms and their products present in an experimental animal can be collected (10–15 per cent for the anthrax infection described below). Large volumes of infected exudate were desirable in the thoracic and peritoneal cavities to facilitate removal of the maximum amount of material. Initial infection was therefore carried out in these cavities. To obtain the maximum yield of products, the largest available animal, *e.g.*, a sheep, would have been the best host for the infection, but, if the ultimate products of chemical extractions are to be adequately tested biologically in a homologous system, a species available in quantity has to be used. The guinea-pig was a successful compromise between these two requirements.

The purity of extracellular products from *in vivo* cultures must be low, since they are grossly contaminated with plasma constituents. It is hoped that chemical fractionation will remove these impurities. As regards the bacteria, however, the possibility of obtaining them almost free of blood cells presented itself. For subsequent chemical fractionation of bacterial extracts a contamination of less than 1 per cent of blood-cell substance could reasonably be called negligible ; it would probably be diluted out in most extraction procedures. Since the degree of contamination with blood cells was to be followed by haemocytometer counts during the differential centrifugation, the relative sizes of blood cells to the chains (4–8 bacilli) of anthrax organisms were estimated. Figures of 2·5–5 × for red blood cells and 8–16 × for white blood cells were obtained. This means that haemocytometer counts on the final product must indicate a contamination of less than 0·05 per cent for each type of cell, if the limit of purity stated above is to be maintained. For organisms smaller than these bacterial chains this figure would have to be less than 0·005–0·001 per cent.

To obtain this degree of purity careful attention must be given to the details of the method described below, especially those dealing with the removal of supernatants from lightly packed blood cell deposits.

METHODS AND RESULTS.

Abbreviations—red blood cell (R.B.C.), white blood cell (W.B.C.), chain of anthrax organisms (B.C.).

A Method used for the Collection and Separation of B. anthracis *and its Products from Infected Guinea-pigs.*

The experimental disease.

One hundred guinea-pigs (800–1000 g.) were given simultaneous intrapulmonary and intraperitoneal injections of 1×10^8 *B. anthracis* spores. The strain used (N.P.) was a non-proteolytic mutant of the Vollum strain obtained by Wright, Hedberg and Feinberg (1951).

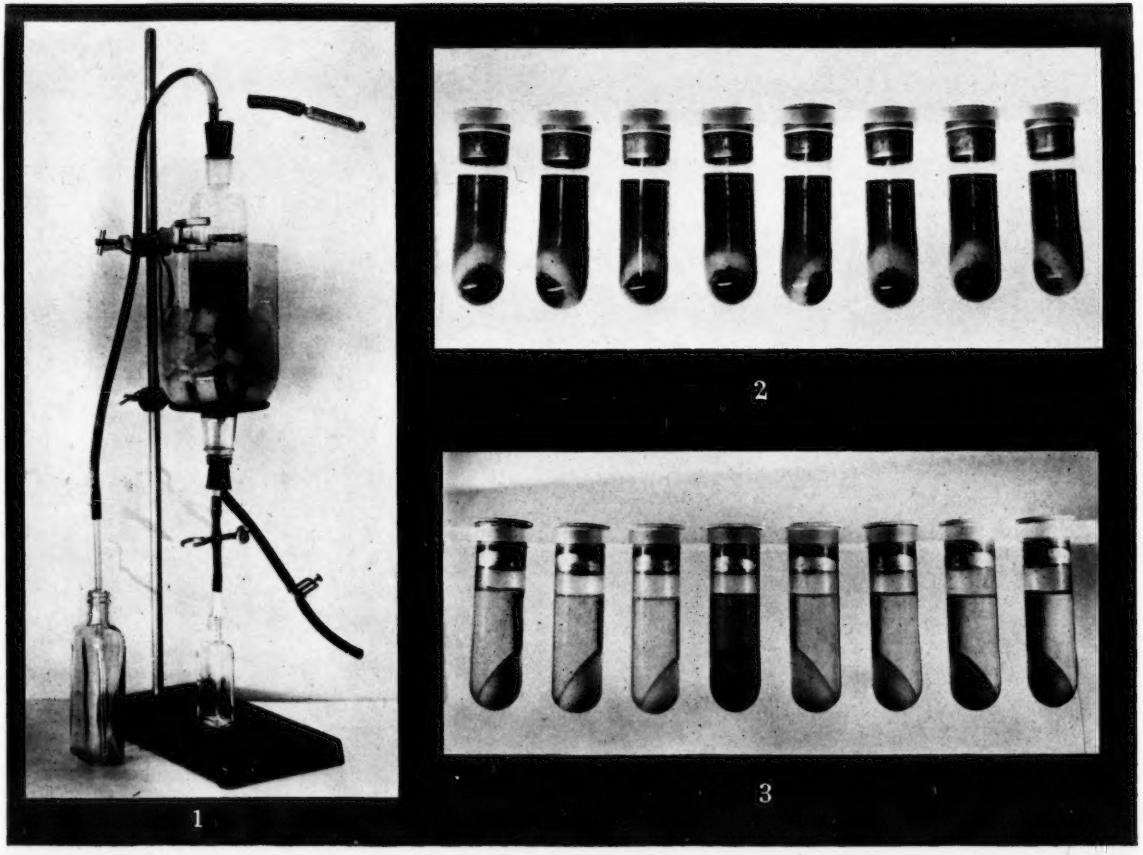
DESCRIPTION OF PLATE.

FIG. 1.—Apparatus for collection of supernatants and for their re-distribution into fresh centrifuge pots.

FIG. 2.—The deposit after high-speed centrifugation of successive batches of material showing the single layer of blood cells beneath the bacteria.

FIG. 3.—The final bacterial deposits together with the blood cells which have been removed in the washing procedure.

BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY. Vol. XXXIV, No. 5.



1

2

3

Smith, Keppie and Stanley.

It was selected from a group of 10 virulent strains which were tested in preliminary experiments for their ability to produce large volumes of thoracic and peritoneal exudate. A dense stock suspension of heated spores was stored at 0° and was the single source of *B. anthracis* employed throughout this work. All the 100 guinea-pigs infected in this manner died 36–60 hr. later. As the main septicaemia begins within a few hours of death and increases hourly the body fluids were collected immediately after death.

Collection of infected body fluids.

The dead animal was secured on a sloping board with the head higher than the hind quarters. This allowed the exudate to drain to the bottom of the cavities and aided its subsequent removal. The chest was opened and the exudate (15–30 ml.) collected in a syringe and expelled into centrifuge pots (250 ml.) standing in ice. The latter had caps made of lint to prevent the escape of aerosol, and they contained sufficient sodium citrate to give a final concentration of 0·6 per cent w/v. The blood was collected after the chest had been opened and the lungs removed, by puncturing the heart and severing the veins and arteries. The blood and the peritoneal exudate were added to the thoracic exudate. Each animal gave 20–30 ml. of this mixture which contained $1{\cdot}2–2{\cdot}6 \times 10^8$ chains of anthrax bacilli (4–8 per chain) per ml.

Low temperature manipulation of the material.

As described above, the infected body fluids were cooled in ice immediately after collection. From then on all the procedures described below such as centrifugation and filtration were carried out at 0–3°. Fig. 1 shows an apparatus which simplified the careful removal of large volumes of supernatants from cell deposits. The material was drawn into the ice-cold reservoir by a slight negative pressure produced by a water pump. It could then be dispensed into fresh centrifuge pots when necessary.

Separation of the bulk of the blood cells from bacteria by differential centrifugation.

The infected body fluids were processed continuously as they were collected. Three full centrifuge pots (250 ml.) constituted the first batch and two pots each subsequent batch.

First slow centrifugation.—The initial material was centrifuged at 500 r.p.m. (60 **g**) for ½ hr. The supernatant containing the bacteria was removed to within ½ in. (1·25 cm.) of the blood-cell deposit and distributed into two fresh pots (250 ml.). The impure supernatant remaining was added to the next sample of initial material. The deposit of blood cells was discarded.

Second slow centrifugation.—The supernatant was again centrifuged at 500 r.p.m. for ½ hr. Only two-thirds of the supernatant was removed to avoid disturbing the small blood-cell deposit. The same two pots were used for this centrifugation of subsequent batches. When all the infected fluids had been treated, the material remaining in these two pots (160–180 ml., B.C. count $2{\cdot}3–2{\cdot}7 \times 10^8$/ml.) constituted 10–15 per cent of the total yield. To avoid losing this material and to reduce its content of blood cells to the required level it was twice centrifuged at 500 r.p.m. for ½ hr. Each time almost all the supernatant was carefully removed from the cell deposits which were discarded.

High speed centrifugation.—The supernatant was centrifuged at 10,000 r.p.m. (12,000 **g**) for 20 min. in the sealed head of an angle centrifuge. The supernatants of plasma/exudate were removed but the bacteria from 100 guinea-pigs were accumulated in the same tubes.

The cell counts recorded in Table I show the progressive removal of blood cells by this differential centrifugation without loss of bacteria.

Removal of the remainder of the blood cells while washing the bacterial deposit.

At the end of differential centrifugation the total bacteria were contaminated with 1·4 per cent of R.B.C. and 0·2 per cent of W.B.C. As successive batches were centrifuged the blood cells did not form a layered arrangement with the bacteria as one might have expected, but slipped through the deposit to form a single red-cell layer at the bottom of the tube. Fig. 2 shows the cell deposits at this stage in the process.

The bacteria were freed from adhering plasma/exudate by washing them three times with Locke's solution, and during this process the remaining blood cells were removed in the following manner. By the careful use of a Pasteur pipette, first on the undiluted sludge

TABLE I.—*Summary of Cell-counts during Differential Centrifugation of Infected Blood and Exudate.*

Nature of sample.	Bacterial chains: Number per ml. ($\times 10^8$).	Percentage contamination with blood cells. R.B.C.	W.B.C.
Initial material	1·7 (1·2–2·6)	Large numbers	Large numbers
a. First slow centrifugation:			
Supernatant	2·0 (1·4–3·2)	4 (3–6)	0·6 (0·4–1·0)
Deposit	0·3 (0·1–0·6)	Large numbers	Large numbers
b. Second slow centrifugation:			
Supernatant	1·8 (1·3–2·5)	1·4 (0·7–3·0)	0·2 (0·06–0·4)
c. Removal of bacteria by high speed centrifugation:			
Supernatant	0·001 (0·0005–0·002)	*nil*	*nil*

These figures are a summary of haemocytometer counts for B.C., R.B.C., and W.B.C. carried out on 12 different batches of material; average figures are quoted and the range is given in brackets.

and then with the use of a little of the washing fluid, most of the bacteria could be removed from the red cell layer. The bacteria were distributed into 7 tubes and the blood cells containing some adhering bacteria were collected into one tube. The material in all tubes was then washed with Locke's solution (approx. 30 ml. per tube). After high-speed centrifugation as described above the supernatants were collected and more bacteria could now be removed from the single red layer. This procedure was repeated twice. After the final washing, few bacteria remained to be removed from the blood cells, which were discarded (see Fig. 3).

The counts recorded in Table II confirm the success of the washing procedure in removing the remaining blood cells to give the final product.

TABLE II.—*Summary of Cell-counts during the Removal of Remaining Blood Cells when the Bacteria were Washed.*

Nature and Volume of sample.	Bacterial chains: Number per ml. ($\times 10^9$).	Percentage contamination with blood cells. R.B.C.	W.B.C.
Initial material (approx. 21.)	0·18 (0·13–0·25)	1·4 (0·7–3·0)	0·2 (0·06–0·4)
Final material (225 ml.)	2·1 (1·7–2·4)	0·02 (0·01–0·03)	0·02 (0·005–0·03)
The blood-cell layer (10 ml.)	0·4 (0·18–1·0)	Large numbers	Large numbers

These figures are a summary of haemocytometer counts for B.C., R.B.C., and W.B.C. carried out on 6 different batches of material; average figures are quoted and the range is given in brackets.

Treatment and total yield of plasma/exudate and bacteria.

The plasma/exudate collected from the high-speed centrifugation was sterilised by filtration. Berkfeld candles originally used have recently been replaced by "Millipore" filters of the Lovell Chemical Company, Watertown 72, Massachusetts, U.S.A., with excellent results. They are composed of cellulose acetate and are of the thickness of normal laboratory filter-paper; hence there is no possibility of large scale absorption of products. To obtain a good filtration rate, the somewhat viscous plasma/exudate was diluted with an equal quantity of Locke's solution.

The bacterial sludge was immediately extracted by the methods described later. The three washings of the bacteria were collected separately from the plasma/exudate since they might contain materials washed from the surface of the bacteria.

Table III summarizes the yield of plasma/exudate and bacteria obtained from five different batches of 100 guinea-pigs. The small loss of bacteria in the total discarded blood cell deposits is included.

TABLE III.—*Yields of Plasma/exudate and* B. anthracis *Obtained from Batches of 100 Guinea-pigs.*

Initial material from 100 guinea-pigs.	Recovered plasma/ exudate.	Recovered bacteria (Volume of final suspension 225 ml.).		Discarded R.B.C./ W.B.C. deposits.		
		Number ($\times 10^9$) of B.C. per ml.	Wt. (g.) dried bacterial extract* obtained.	Vol. (ml.).	Number ($\times 10^8$) B.C. per ml.	Loss of B.C. as per cent of recovered B.C.
(B.C. count 1·2–2·6 $\times 10^8$ per ml.) Vol. (l.).	(Vol. (l.).)					
2·8	2·3	1·8	2·2	500	—	—
2·2	1·8	1·7	1·8	400	1·0	11
2·2	1·7	1·7	1·7	450	0·9	11
3·0	2·5	2·2	2·1	500	1·3	13
3·2	2·6	2·2	2·5	600	1·1	13

* For details of preparation see next paper.

Application of the Above Method to the Products of Other Infections.

To test whether the method could be generally applied to obtain bacteria of sufficient purity, small numbers of guinea-pigs were infected with five different organisms. These were recently isolated strains of *Streptococcus pyogenes* (2 strains), *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Listerella monocytogenes* and a virulent strain of *Past. pestis*. Dense suspensions of the first five organisms were prepared by washing off cultures on blood agar with Locke's solution. *Past. pestis* was grown on one tryptic meat agar slope, washed off with Locke's solution (10 ml.) and diluted 1/100. Each suspension was injected intrapulmonarily (2 ml.) and intraperitoneally (8 ml.) into two guinea-pigs (over 800 g.); the *Str. pneumoniae* suspension was mixed with an equal amount of 2 per cent w/v hog gastric mucin. All the animals died in 20–24 hr. and with the exception of those infected with *List. monocytogenes*, contained large numbers of organisms in their body fluids. The thoracic exudate, peritoneal exudate (washed out with Locke's solution (10 ml.) containing 0·6 per cent citrate) and blood were collected, and treated as described above. The cell counts recorded in Table IV show that blood-cell contamination is very low at the end of differential centrifugation. Counts were difficult on the final deposits owing to the presence of clumps formed on high-speed centrifugation. These counts are not included in Table IV, but they indicated an even lower contamination with blood cells.

TABLE IV.—*Cell-counts after Differential Centrifugation of the Products Harvested from Other Infections in Guinea-pigs.*

Organism producing the infection.	Number (per ml.) of cells present after differential centrifugation.		
	Bacteria.	R.B.C.'s ($\times 10^4$).	W.B.C.'s ($\times 10^4$).
Str. pyogenes (1)	8×10^6	Less than 1	Less than 1
,, ,, (2)	4×10^8	,, ,, 2	,, ,, 1
Str. pneumoniae	1×10^8	,, ,, 1	,, ,, 1
Staph. aureus	2×10^9	,, ,, 1	,, ,, 1
Past. pestis	3×10^9	,, ,, 3	,, ,, 3
List. monocytogenes	1×10^6	,, ,, 1	,, ,, 1

The products (approx. 50 ml.) were the bulked thoracic exudate, peritoneal washings and blood from two guinea-pigs (over 800 g.) infected with the organism under test.

DISCUSSION AND SUMMARY.

Using *B. anthracis* in the guinea-pig, a method has been established for the separation of bacteria and their products from infections in experimental animals. It has been possible to collect from 100 infected guinea-pigs 1·5–2·0 g. of *B. anthracis* and 1·5–2·5 l. of body fluids containing the extracellular products of this organism. These quantities were sufficiently large to allow a chemical study of the factors involved in the virulence of *B. anthracis* in guinea-pigs (see following paper).

This is the first time that large quantities of bacteria have been collected from culture *in vivo*. A high degree of freedom from blood cells has been obtained by a process involving only a small overall loss (10–13 per cent) of the total bacteria collected. The maximum contamination (0·03 per cent) with both types of blood cell was well below the desired limit indicated in the introduction to this paper.

As regards the almost complete separation of blood cells from bacteria, this method has been shown to be equally effective for the body fluids obtained from five other infections in guinea-pigs. Since the experiments were on a small scale, no definite information other than the counts given in Table IV has been gained on the amount of material that could be obtained from these infections.

Our thanks are due to Dr. D. W. Henderson and Prof. W. T. J. Morgan for encouragement, particularly in the early stages of this work. We are also indebted to Mr. T. Snape for excellent technical assistance, to our colleague Mr. H. T. Zwartouw for the photographs, and to the M.O.S. Animal Farm for the supply of guinea-pigs.

Acknowledgment is made to the Chief Scientist, Ministry of Supply, for permission to publish this paper.

REFERENCES.

Cromartie, W. J., Watson, D. W., Bloom, W. L., and Heckly, R. J.—(1947) *J. infect. Dis.*, **80**, 14.

Jawetz, E. J., and Meyer, K. F.—(1943) *Ibid.*, **73**, 124.

Pollitzer, R.—(1952) *Bull. World Health Org.*, **5**, 73.

Smith, H., Keppie, J., and Stanley, J. L.—(1953) *Brit. J. exp. Path.*, **34**, 477.

Wilson, G. S., and Miles, A. A.—(1948) 'Topley and Wilson's Principles of Bacteriology and Immunity.' London (Edward Arnold & Co.), p. 775.

Wright, G. G., Hedberg, M. A., and Feinberg, R. J.—(1951) *J. exp. Med.*, **93**, 523.

THE CHEMICAL BASIS OF THE VIRULENCE OF *BACILLUS ANTHRACIS*. I: PROPERTIES OF BACTERIA GROWN *IN VIVO* AND PREPARATION OF EXTRACTS.

H. SMITH, J. KEPPIE, AND J. L. STANLEY.

From the Microbiological Research Department, Porton, nr. Salisbury, Wilts.

Received for publication April 29, 1953.

STUDIES on the purification and chemical identification of bacterial products have been related mostly to their immunising action and their serological behaviour. Less attention has been given to the part played by these products in the disease process and in causing the death of the host. This is particularly true of those organisms capable of producing septicaemic conditions in which no potent extracellular toxins seem to play a part. The research now undertaken is directed towards chemical identification of substances and processes connected with the virulence of organisms producing such septicaemic diseases.

The virulence of a bacterium is best defined as its capacity to infect the host and establish disease. To do so the organism must overcome the defence mechanisms of the host. The course of the disease is influenced by the strength of the latter, which may affect the metabolism of the organism. There is abundant evidence that the complex metabolic activity of a pathogen in establishing disease is different from its metabolism *in vitro*. The particular nutritional conditions of the host tissues undoubtedly play their part, but the possibility that the organism reacts in some way to the challenge of the host's defence mechanisms must be borne in mind.

Our limited knowledge of bacterial nutrition makes it impossible at the moment to reproduce *in vitro* the conditions of growth *in vivo*. It seemed therefore that the best way of studying compounds and reactions responsible for virulence was to use organisms and their products isolated from infected animals in spite of the technical difficulties involved. This does not necessarily mean, as Bail and Weil suggested (1911), that there is an absolute difference between "animal organisms" and those grown *in vitro*. If the compounds and reactions responsible for virulence in the host could be identified, it might lead to the production in defined media of some of the metabolic processes which occur *in vivo*. The recent preparation of the immunising antigen of *Bacillus anthracis* by artificial culture (Gladstone, 1946, 1948) is encouraging in this respect.

It was therefore decided to carry out work on the chemical basis of virulence, using as starting materials the bacteria and their products from infected animals. *B. anthracis* was chosen as the pathogen to be studied for two main reasons. In the first place anthrax is characterised by the presence in the host of large numbers of organisms, and this made it probable that the yield of bacteria would be satisfactory. Secondly, the extracts from the literature given below show that the precise chemical basis of invasiveness, killing power and immunity to the disease is unknown. However, sufficient biological knowledge has been gained to indi-

indicate the general lines of attack on these problems and the probable value of materials harvested from growth *in vivo*.

Reviews of the literature on the pathology and immunochemistry of *B. anthracis* and its infections are available (Sobernheim, 1913, 1931 ; Cromartie, Bloom and Watson, 1947 ; Treffers, 1947). Only the main outlines of present knowledge of the disease are mentioned here, and it will be seen that attention has been focussed on three main topics.

Immunity to anthrax has received much attention. For nearly half a century following the use of live attenuated cultures by Pasteur (1881), and sterile oedema fluid from infected animals by Bail (1904), for successful vaccination against the disease, no further progress was made. Dead bacteria and various bacterial extracts (Gladstone, 1946) were ineffective. Gladstone (1946, 1948) however using *ad hoc* methods produced an extracellular immunising antigen by *in vitro* culture, a fact which has been confirmed (Heckly and Goldwasser, 1949 ; Wright, Hedberg and Feinberg, 1951). This protective antigen has not yet been connected by experimental evidence with any of the usual serological reactions (Watson, Cromartie, Bloom, Kegeles and Heckly, 1947), or with any aspect of virulence, although Bail (1904) suggested its identity with the aggressin. Its chemical nature is unknown and it does not appear to be connected with either of the two known antigens of *B. anthracis* (Staub and Graber, 1943 ; Staub, 1949), the D-polyglutamic acid of Ivanovics and Bruckner (1937) or the somatic polysaccharide of Ivanovics (1940). Watson, Cromartie, Bloom, Kegeles and Heckly (1947) found that immunising activity was associated with the globulin fraction of rabbit oedema fluid.

With regard to the ability of *B. anthracis* to grow in the host tissues, Bail and Weil (1911) found that anthrax lesions contain substances which interfere with host resistance. These substances were termed aggressins, and together with similar substances from other organisms formed the basis of the much discussed " aggressin theory " (see Wilson and Miles, 1948). Gruber and Futaki (1907) and Preiss (1909) suggested that these substances were associated with the formation of the capsule, because crude capsular material protected the organism from phagocytosis and the bactericidal action of certain body fluids. Bail and Weil (1911) did not think that the aggressin and the capsule were connected. No chemical study has progressed far enough to correlate these aggressins with one or more definite chemical compounds, but Watson, Cromartie, Bloom, Heckly, McGhee and Weissman (1947) showed that their crude " inflammatory factor " inhibited completely the anthracidal substance from leucocytes and other tissue cells.

Finally, the cause of the death of the host has received some attention, but no definite conclusions have been reached. The main property of the crude " inflammatory factor " of the above workers was its ability to produce the oedematous tissue damage characteristic of the disease, but it was not grossly toxic. This property and its anticoagulant activity showed the difference between this preparation, which contained a high proportion of glutamic acid, and the D-polyglutamic acid of *Bacillus subtilis*. Eurich and Hewlett (1930) and Sobernheim (1931) regarded the presence of a definite toxin as unproven, and some recent work by King and Stein (1950) supports this view. Several different workers agree that a marked hyperglycaemia occurs in anthrax infection (Cromartie *et al.*, 1947 ; Bloom, McGhee, Cromartie and Watson, 1947). An interference with

calcium metabolism is suggested as a possible cause of death by the work of Bloom *et al.* (1947). The symptoms of the disease were compared with those of calcium deficiency, and the protective effect of certain calcium salts was noted. Deoxygenation of the blood and mechanical capillary obstruction have also been put forward as possible explanations, but have not yet been substantiated (Cromartie *et al.*, 1947 ; Bloom *et al.*, 1947). De Moulin (1936) noted lesions in the nervous system in anthrax, and attributed the symptoms of the disease and the death of the host to damage of this system.

In a previous paper (Smith, Keppie and Stanley, 1953) a method is described for the collection and separation of *B. anthracis* and body fluids containing its extracellular products from infected guinea-pigs in quantities of 1·5–2·0 g. and 1·5–2·51 respectively, from 100 animals. The bacteria were almost free from blood cells (not more than 0·03 per cent), and the quantity of these products was sufficient for a chemical approach to the question of virulence of *B. anthracis* in guinea-pigs.

The sterile extracellular products, although grossly contaminated with plasma constituents, could be examined directly for biological properties connected with virulence, and undergo chemical extraction for the substances responsible for these biological properties. Before the same procedure could be adopted for the bacterial substance two questions remain to be answered. First, were these bacteria appreciably different from those obtained by growing the same strain under a variety of conditions *in vitro* ? Secondly, could sterile extracts containing nearly 100 per cent of the original bacterial substance be prepared by mild methods ? The work described in this paper gives an affirmative answer to both questions.

METHODS AND RESULTS.

In these experiments the strain of *B. anthracis* (N.P.) and the breed of guinea-pig used were those employed in the infection from which the bacterial products were obtained (Smith *et al.*, 1953).

The Difference between B. anthracis *from Infected Guinea-pigs and those Cultivated* in vitro.

The organisms from infected guinea-pigs were shown to be different from those cultivated *in vitro* by a combination of three criteria. Two of these, morphology and susceptibility to phagocytosis, are well known. The behaviour of the organisms in a dilute solution of ammonium carbonate however has not been previously described.

For this comparison *B. anthracis* was grown *in vitro* in four different media—tryptic meat broth (T.M.B.), a modified medium of Brewer, McCullough, Mills, Roessler and Herbst (1946) (B.M.), sheep serum (S.S.), and citrated guinea-pig plasma (G.P.P.). Using turbidity measurements as a guide, samples were taken at four different times—at the beginning, the middle and the end of the phase of rapid growth and at 24 hr. Table I gives the details of the preparation of these cultures. The bacteria were collected by centrifugation (12,000 g) at 0°, and washed with Locke's solution as described for the organisms obtained from infected guinea-pigs.

Morphology and susceptibility to phagocytosis.

Bacteria isolated from infected guinea-pigs had well-developed capsules, and were completely resistant to the action of guinea-pig phagocytes in the system

TABLE I.—*Turbidity Measurements during the Growth of* B. anthracis *in Different Media.*

Medium.*	Time shaken at 37° (hr.).												
	0.	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	12.	24.
T.M.B.	0·04	0·05	0·17	0·44	0·95	1·06	1·14	1·18	—	—	—	—	1·06
B.M.	0·14	—	0·10	—	0·12	0·26	—	0·48	0·75	0·93	0·95	0·88	0·71
S.S.	0·07	—	—	—	0·08	0·22	0·35	0·53	0·72	0·83	0·82	0·81	0·86
G.P.P.†	0·12	0·25	0·33	0·45	0·60	0·79	0·99	—	1·4	—	—	—	2·0

Initial inoculum 3×10^7 spores per ml. of medium. Figures are turbidity readings (—log percentage transmission at wave length 610 mμ.) compared with a blank of the original medium.

The time (hr.) at which the four samples of cultures were taken for comparison with organisms grown *in vivo* by the methods described in the text were as follows: T.M.B. 3, 3½, 5, 24; B.M. 6½, 7½, 10, 24; S.S. 6½, 8, 10, 24; G.P.P. 3, 5, 8, 24.

* For definition of media see text.

† Spores previously germinated in tryptic meat broth.

described in the following paper (Keppie, Smith and Harris-Smith, 1953). Young organisms from T.M.B. cultures were almost devoid of capsule and had no resistance to phagocytosis. Young organisms from the other three media had capsules which varied in width according to the medium, but were smaller than those of organisms from animals. They also resisted phagocytosis except for a small minority of cells which appeared to be degenerate and had lost their capsule.

It is true therefore for the strain N.P. as for other virulent strains of *B. anthracis* that the organisms from an infected host have better-developed capsules and are more completely resistant to phagocytosis than are most organisms grown *in vitro*.

The action of 0·16 *per cent w/v ammonium carbonate solution.*

B. anthracis isolated from guinea-pigs dissolve readily in ammonium carbonate solution. This property has been used to distinguish these organisms from the 16 samples which were grown *in vitro* and collected as described above.

The washed organisms from 20 ml. of culture were immediately homogenised with ice-cold distilled water (20–22 ml.). This suspension (9·6 ml.) was placed in two spectrophotometer tubes. To one tube 4 per cent w/v ammonium carbonate solution (0·4 ml.) was added, and to the control the same amount of water. The tubes were kept at 0–3°, and at various time intervals turbidity readings (— log per cent transmission of light of wave length 610 mμ.) were taken using a distilled water blank. Any lysis of the organisms in 0·16 per cent w/v ammonium carbonate solution was thus detected.

It was important that the amount of organisms treated with ammonium carbonate should be of the same order in all samples. Although the amounts of organisms in 20 ml. of the cultures described above were not identical; they all produced a turbidity reading within the range 0·4–0·8 before the addition of ammonium carbonate. The amounts of bacteria isolated from guinea-pigs and used in five comparison experiments were selected so that their initial turbidity readings extended over this range. Variation of initial turbidity within this range did not significantly affect the degree of lysis. The figures quoted in Table II are the averages of the five experiments.

Table II summarises the results of these experiments. In most of them there was a slight change in the turbidity reading of the control as the experiment proceeded. For the sake of brevity in presenting a large number of comparisons of experiment with control, the figures quoted are the differences between the reading of the control, and that of the experiment expressed as a percentage of the former, *e.g.*, control 0·67, experiment 0·46, figure quoted 31; control 0·65,

TABLE II.—*The Action of* 0·16 *per cent Ammonium Carbonate Solution at* 0° *on* B. anthracis *grown* in vivo *and* in vitro.

Sample of *B. anthracis.**		Time of readings (hr.). 1.	2.	3.	4.	5.	6.	7.	8.	24.
Organisms isolated from guinea-pigs		13	24	36	44	51	61	67	72	86
T.M.B.	(1)	1	3	3	5	8	16	25	35	85
	(2)	0	1	3	4	5	7	11	15	73
	(3)	0	0	0	0	0	0	2	4	33
	(4)	0	2	5	0	1	4	4	4	18
B.M.	(1)	0	0	0	2	2	2	2	2	11
	(2)	0	0	0	0	0	0	0	0	13
	(3)	0	2	0	0	0	0	0	0	3
	(4)	2	1	2	2	1	2	2	3	0
S.S.	(1)	0	2	4	5	5	5	5	5	22
	(2)	0	0	3	3	0	0	0	0	24
	(3)	0	0	0	0	0	0	0	0	5
	(4)	0	0	0	0	0	0	0	0	5
G.P.P.	(1)	1	4	4	5	5	6	7	8	14
	(2)	2	2	2	4	4	5	5	6	21
	(3)	2	4	5	8	8	8	8	8	12
	(4)	0	2	6	6	6	9	8	8	8

The figures are a measure of the lysis of organisms in ammonium carbonate solution, 0 indicating no lysis ; they are the difference between the turbidity readings (– log percentage transmission at wave-length 610 mμ.) of the control and those of the experiment, expressed as a percentage of the former.

* For details of preparation see Table I.

experiment 0·17, figure quoted 74. All figures are the average of two or more experiments, and are a measure of the lysis of the organisms in the dilute ammonium carbonate solution.

The rapid solution of the *in vivo*-grown organisms is not shown by the 16 samples of organisms grown *in vitro* even by those obtained from media containing plasma constituents.

Preparation of Sterile Bacterial Extracts by Mild Methods.

In these experiments the washed *B. anthracis* from infected guinea-pigs were used.

Attempts to remove the capsule from B. anthracis.

The evidence that the capsule of *B. anthracis* is composed of polyglutamic acid consists solely of the fact that non-capsulated organisms produce none of this material. When a capsule is formed there is no reason to suppose that it consists entirely of polyglutamic acid. Indeed Tomcsik (1951) has put forward some evidence for the presence of polysaccharide in the capsule. A preferential removal of the capsule would have been of great value in these studies in view of the possible connection of the capsule with virulence (Treffers, 1947). Following the publication of the work of Amies (1951) on the removal of the capsule from *Pasteurella pestis* by solution of KCNS, in which it is mentioned that the same method worked for *Bacillus mycoides*, considerable attention was devoted to this problem, but without success.

The classical method of wet India ink preparations was used to indicate the presence of a capsule. The following substances were examined in various concentrations both at room temperature and at 0° to see if they would preferentially remove the capsule :—KCNS, KI, NH_4CNS, sodium salicylate, guanidine hydrochloride, urea, ammoniacal copper sulphate, ammonium acetate, mono-, di- and triethanolamine, tergitol, sodium taurocholate, sodium desoxycholate, formamide, ethylene glycol, diethylene glycol, pyridine, ether water, chloroform water, dilute ammonia solutions, Na_2CO_3, NaOH and acetic acid. Not one had the desired effect.

Although no clear-cut removal of the capsule could be effected, stained films of *B. anthracis* grown *in vivo* show that some capsular material tends to become detached from the organism. This is supported by the fact that chemical fractionation of the plasma/exudate has revealed the presence of polyglutamic acid. There is a distinct possibility that the three washings of the bacteria with Locke's solution (Smith *et al.*, 1953) may have removed some capsular material. These washings have been kept separate for chemical and biological examination.

A bacterial extract prepared by the action of 0·16 *per cent w/v ammonium carbonate.*

It was noticed in the above experiments that 50 per cent aqueous pyridine and 0·1 N ammonia solution dissolved the organisms completely at 0°. This was not solely a pH effect since $NaHCO_3$, Na_2CO_3 and NaOH solutions did not give the same degree of lysis.

Concentrating on the action of the ammonium ion rather than that of pyridine, experiments were designed to find the lowest pH at which this ion would effect complete solution of the organisms. Mixtures of ammonia, ammonium carbonate and ammonium acetate in dilute solution were used. A 0·16 per cent w/v ammonium carbonate solution (pH 8·8) was the most satisfactory, and after the organisms had dissolved the pH of the solution dropped to a minimum of 8·3. Mixtures of ammonium carbonate and ammonium acetate with reactions below pH 8·2 did not dissolve the organisms. Spores were not affected. After lysis the viable count corresponded to the few spores present in the original suspension.

The procedure for preparing the extract is as follows : The sludge (50 ml.) of bacterial chains ($3–5 \times 10^{11}$) from 100 guinea-pigs was transferred in ice-cold distilled water to a bottle (900 ml.) containing glass beads (100 ml.). Ammonium carbonate solution (4 per cent w/v, 32 ml.) was added and the bottle filled with water and shaken at 0–3° overnight. The solution was clarified from a slight turbidity by centrifugation at 0–3° for 15 min. at 10,000 r.p.m. (12,000 *g*) in a refrigerated centrifuge. After filtering through sintered glass filters and testing for sterility the extract was freeze-dried. The small residue from centrifugation was re-extracted with 0·16 per cent w/v ammonium carbonate solution (80 ml.).

Table III shows the details of five routine preparations of this extract in which the yield of insoluble material is also given. This material, which was autoclaved, freeze-dried and finally dried at 0·1 mm. Hg and at 60° before weighing, consisted of spores and bacterial débris.

A totally unexpected advantage of this method was the sublimation of the ammonium carbonate during freeze-drying. Determination of ammonia as ammonium salts in 7 different freeze-dried preparations corresponded to 1–2 per cent of ammonium carbonate. This small quantity of ammonia is probably present as the ammonium salts of other acids.

TABLE III.—*The Extraction of* B. anthracis *with Ammonium Carbonate Solution and by Shaking with Ballotini.*

Nature of extract (vol. approx. 800 ml.).	Initial material (from 100 guinea-pigs). Total No. (× 10^{11}) of B.C.	Soluble material: pH of filtered extract.	Soluble material: Wt. (g.) of freeze-dried extract.	Soluble material: *Wt. (g.) of extract corrected for moisture and solids of the Locke's soln. in bacterial sludge.	Insoluble material: Dry wt. (g.).	Insoluble material: Percentage of soluble material.
Ammonium carbonate solution (0·16 per cent w/v).	4·0	8·3	2·2	1·8	0·087	4·8
	3·8	8·5	1·8	1·4	0·062	4·4
	3·8	8·7	1·7	1·3	0·055	4·2
	4·9	8·7	2·1	1·7	0·075	4·4
	4·9	8·7	2·5	2·1	0·080	3·8
Ballotini extract	4·5	7·1	1·7	1·3	0·098	7·5
	5·4	6·9	2·2	1·8	0·075	4·2
	4·1	6·8	1·8	1·4	0·070	5·0
	5·1	6·7	2·0	1·6	0·061	3·8

* Moisture: Loss at 0·1 mm. Hg and 60°. Vol. of sludge is 50 ml.; the weight of the solids of the Locke's solution in the sludge cannot be more than 0·5 g., and is estimated to be 0·25 g.

A bacterial extract prepared by shaking with ballotini at 0°.

A more conventional method of extraction was that used by Curran and Evans (1942) and King and Alexander (1948). The organisms were shaken with water and "ballotini" Grade 12, *i.e.*, minute glass beads with an average diameter of 0·13 mm. (Messrs. Chance Bros., Smethwick, England). After preliminary experiments to find the best proportions of ballotini, distilled water and bacterial sludge the following procedure was used:

The bacterial sludge from 100 guinea-pigs was transferred with ice-cold water to a bottle (1150 ml.) containing ballotini (350 ml.). The bottle was filled with distilled water and shaken 24 hr. at 0–3°, only a small air bubble being left so that foam (and denaturation) was reduced to a minimum. The extract, the pH of which hardly varied from 7·0, was treated in the same manner as the ammonium carbonate extract. The re-extraction of the residue was done with water (80 ml.) and ballotini (35 ml.).

Table III includes the details of four routine preparations in which the yield of insoluble material (treated as described above) is quoted. This material consisted of spores and bacterial debris.

Results of freezing and thawing experiments.

By freezing the diluted bacterial sludge at −20°, then allowing it to thaw at room temperature and repeating the process 15–20 times, the viable count of the sample dropped to 10^2–10^3/ml. However, the samples after this procedure remained grossly turbid, and there was no hope of sterile filtration yielding a soluble extract containing the same high proportion of bacterial substance as in the previous two methods. This method was therefore abandoned.

DISCUSSION.

The widely held view that organisms from an infected host differ from those obtained from cultures has been substantiated for *B. anthracis* (N.P.) from infected guinea-pigs. A study of morphology and susceptibility to phagocytosis sharply differentiated organisms grown *in vivo* from those grown in broth, but not from those cultivated in the other three media which contained some plasma constituents. However, the rapid lysis of organisms from guinea-pigs in ammonium carbonate solution was not shown by any of the organisms grown *in vitro* including those from media containing plasma constituents. This lysis, the basis of which is unknown, serves to underline the chemical difference between these organisms.

After the failure of attempts to remove the capsule of *B. anthracis*, two methods, the use of ammonium carbonate solution and shaking with ballotini have been used to dissolve the bacterial substance with excellent results. The proportion of material dissolved was over 95 per cent. Of the two methods, extraction with dilute ammonium carbonate solution was easier to carry out, and it is doubtful whether any gross harm was done to constituents by a pH of 8·8–8·3 at 0–3°. However, the second method of extraction at pH 7·0 rules out any possible detrimental effect of these slightly alkaline conditions.

Future work on the chemical basis of virulence of *B. anthracis* in guinea-pigs will follow three main lines. Plasma/exudate (extracellular products) and both bacterial extracts will be examined in biological tests connected with virulence. The crude materials will then be examined chemically to identify the substances responsible for particular biological activity. This involves the extension of the above tests into biological assays for fractionation samples. Finally, observations on the metabolism of *B. anthracis* in body fluids may be attempted together with its repercussions on the metabolism of the host. The following paper describes the first of these investigations.

SUMMARY.

B. anthracis organisms harvested from infected guinea-pigs have been shown to be different from those grown under a variety of conditions *in vitro* mainly by their rapid lysis in ammonium carbonate solution.

Sterile bacterial extracts of *B. anthracis* grown *in vivo* have been prepared by two mild methods and contain over 95 per cent of the bacterial substance.

Our thanks are due to Mrs. P. W. Harris-Smith for carrying out the phagocytic tests.

Acknowledgment is made to the Chief Scientist, Ministry of Supply, for permission to publish this paper.

REFERENCES.

AMIES, C. R.—(1951) *Brit. J. exp. Path.*, **32**, 259.

BAIL, O.—(1904) *Zbl. Bakt., Abt. I*, **47**, 270.

Idem AND WEIL, E.—(1911) *Arch. Hyg., Berl.*, **73**, 218.

BLOOM, W. L., MCGHEE, W. J., CROMARTIE, W. J., AND WATSON, D. W.—(1947) *J. infect. Dis.*, **80**, 137.

BREWER, C. R., MCCULLOUGH, W. G., MILLS, R. C., ROESSLER, W. G., AND HERBST, E. J.—(1946) *Arch. Biochem.*, **10**, 77.

CROMARTIE, W. J., BLOOM, W. L., AND WATSON, D. W.—(1947) *J. infect. Dis.*, **80**, 1.
CURRAN, H. R., AND EVANS, F. R.—(1942) *J. Bact.*, **43**, 125.
DE MOULIN, F. W. K.—(1936) *Ned-ind. Bl. Diergeneesk*, **48**, 126.
EURICH, F. W., AND HEWLETT, R. T.—(1930) 'A System of Bacteriology.' London (Medical Research Council), **5**, 439.
GLADSTONE, G. P.—(1946) *Brit. J. exp. Path.*, **27**, 394.
Idem—(1948) *Ibid.*, **29**, 379.
GRUBER, M., AND FUTAKI, K.—(1907) *Münch. med. Wschr.*, **54**, 249.
HECKLY, R. J., AND GOLDWASSER, E.—(1949) *J. infect. Dis.*, **84**, 92.
IVANOVICS, G.—(1940) *Z. ImmunForsch.*, **97**, 402.
Idem AND BRUCKNER, V.—(1937) *Ibid.*, **90**, 304.
KEPPIE, J., SMITH, H., AND HARRIS-SMITH, PATRICIA W.—(1953) *Brit. J. exp. Path.*, **34**, 486.
KING, H. K., AND ALEXANDER, H.—(1949) *J. gen. Microbiol.*, **2**, 315.
Idem AND STEIN, J. H.—(1950) *Ibid.*, **4**, 48.
PASTEUR, L.—(1881) *C. R. Acad. Sci., Paris*, **92**, 429.
PREISS, H.—(1909) *Zbl. Bakt., Abt. I*, **49**, 341.
SMITH, H., KEPPIE, J., AND STANLEY, J. L.—(1953) *Brit. J. exp. Path.*, **34**, 471.
SOBERNHEIM, G.—(1913) ' Handbuch der Pathogenen Mikroorganismen,' 2nd ed. Jena (Gustav Fischer), **3**, 583.
Idem—(1931) *Ibid.*, 3rd ed., **3**, 1041.
STAUB, A. M.—(1949) *Ann. Inst. Pasteur*, **76**, 331.
Idem AND GRABAR, P.—(1943) *C. R. Soc. Biol., Paris*, **137**, 623.
TOMCSIK, J.—(1951) *Experientia*, **15**, XII, 460.
TREFFERS, H. P.—(1947) *Ann. Rev. Microbiol.*, **1**, 263.
WATSON, D. W., CROMARTIE, W. J., BLOOM, W. L., KEGELES, G., AND HECKLY, R. J. —(1947) *J. infect. Dis.*, **80**, 29.
Idem, HECKLY, R. J., MCGHEE, W. J., AND WEISSMAN, N.—(1947) *Ibid.*, **80**, 121.
WILSON, G. S., AND MILES, A. A.—(1948) ' Topley and Wilson's Principles of Bacteriology and Immunity.' London (Edward Arnold & Co.), p. 1068.
WRIGHT, G. G., HEDBERG, M. A., AND FEINBERG, R. J.—(1951) *J. exp. Med.*, **92**, 523.

THE CHEMICAL BASIS OF THE VIRULENCE OF *BACILLUS ANTHRACIS*. II: SOME BIOLOGICAL PROPERTIES OF BACTERIAL PRODUCTS.

J. KEPPIE, H. SMITH AND PATRICIA W. HARRIS-SMITH.

From the Microbiological Research Department, Porton, nr. Salisbury, Wilts.

Received for publication April 29, 1953.

In a previous paper a method is described for collecting and separating large quantities of *Bacillus anthracis* and its extracellular products (plasma/exudate) from infected guinea-pigs. Two sterile extracts of the intracellular material (ammonium carbonate extract and ballotini extract) were prepared by dissolving the organisms almost completely under mild conditions (Smith, Keppie and Stanley, 1953*a*, *b*). These crude preparations have been examined for biological properties connected with virulence as a preliminary to chemical fractionation for the compounds responsible for such activity. This paper describes the behaviour of these preparations in tests for toxicity, tissue-damaging activity, aggressins, and protective antigen. Similar studies on the extracellular products have already been reported (Treffers, 1947), but this is not so for the intracellular products of *B. anthracis* grown *in vivo*. The behaviour of the latter in the biological tests is therefore of special interest.

The results are discussed in relation to anthrax in the guinea-pig.

METHODS AND RESULTS.

In these experiments the strain of *B. anthracis* (N.P.) and the breed of guinea-pigs used were those employed in the infection from which the bacterial products were obtained (Smith *et al.*, 1953*a*, *b*,). Abbreviations used to describe the latter are P.E. (plasma exudate,) A.C.E. (ammonium carbonate extract) and B.E. (ballotini extract).

Non-toxicity of B. anthracis *Products*

Eurich and Hewlett (1930) and Sobernheim (1931) doubted the toxicity of anthrax products, and this was supported by the work of King and Stein (1950). They found that intra- and extracellular products of a moderately virulent strain of *B. anthracis* grown under various conditions *in vitro* were non-toxic to mice in the doses given. The use of extracts of infected mouse spleens gave equally negative results.

In addition to using highly virulent *B. anthracis* harvested from infected guinea-pigs, a more quantitative approach to this question of toxicity was made in the experiments reported below. The aim was to treat guinea-pigs (500 g.) with sterile bacterial extract equivalent to more bacteria than there are in a guinea-pig when it dies of anthrax, and with a volume of P.E. greater than 100 ml. as this is approximately the volume of extracellular fluid in a guinea-pig (500 g.).

The number of bacterial chains (4–8 bacteria) in guinea-pigs dead from anthrax was estimated as accurately as possible. Six guinea-pigs were examined immediately after

death, and haemocytometer counts were carried out on samples of the blood, thoracic and peritoneal exudates, liver, spleen, lung and kidney in two cases. The latter tissues were weighed after being removed from the animal, ground with water and sand in a mortar to form a fine suspension, and then suitable dilutions of these suspensions were taken for counting. Table I gives the number of anthrax bacilli estimated to be present in the whole guinea-pig and in the main viscera. The total counts for each animal in the series were similar.

TABLE I.—*An Estimate of the Number and Location of Anthrax Bacilli in Infected Guinea-Pigs at Death.*

Guinea-pig (500 g.).	Blood (estimated vol. 50 ml.).	Spleen (1·25–4 g.).	Liver (14–23 g.).	Lungs (3·5–4 g.).	Kidneys (5–8 g.).	Peritoneal and thoracic exudate.	Total.
1	2·75	0·7	0·44	1·08	—	—	4·97
2	1·54	0·92	0·40	0·27	—	—	3·13
3	1·89	0·70	0·30	0·18	0·20	0·66 (15 ml.)	3·93
4	2·75	0·93	0·52	0·40	—	—	4·60
5	3·08	0·64	0·64	0·43	—	—	4·79
6	2·73	0·88	0·56	0·12	0·11	0·38 (15 ml.)	4·78

Figures are the total number ($\times 10^{10}$) of bacterial chains (4–8 bacilli) in each tissue as determined by haemocytometer counts.

Since it was intended to inject P.E. in volumes of 100 ml. or more, the tolerance of guinea-pigs (500 g.) to the intraperitoneal injection of large volumes of normal guinea-pig plasma was investigated. Injection of these large volumes was carried out high in the flank to avoid subsequent leakage. Citrated plasma was toxic in the required volumes, but heparinised plasma (or plasma from defibrinated blood) was not toxic up to a limit of 200 ml. (see Table III).

In the collection of *B. anthracis* and its products (Smith *et al.*, 1953*a*) for these toxicity experiments, heparin (10 I.U./ml.) and not citrate was used as the anticoagulant.

Non-toxicity of bacterial extracts.

The bacteria were used immediately after the differential centrifugation procedure and removal of the final P.E. The washing with Locke's solution was omitted to avoid the danger of removing or destroying toxic principles by this treatment. Bacterial counts of this sludge showed that the total quantity of bacteria was appoximately 10 times the number estimated to be present in one guinea-pig (500 g.) when it dies of anthrax. The material was divided into 5 parts, and after the treatments described below, each part was injected into a single guinea pig (500 g.) This meant that each guinea-pig received bacterial extract equivalent to approximately twice the number of anthrax bacilli estimated to be present in a guinea-pig killed by anthrax It is unlikely that the error in the latter estimate is more than twofold.

The total bacterial sludge was homogenised in distilled water (800 ml.) and divided into 5 parts which were treated as follows.

(*a*) *Filtered A.C.E.*—Prepared as described previously (Smith *et al.*, 1953*b*). After filtering, the solution (160 ml. + 40 ml. water used to wash the filter) was adjusted to pH 7·2 with acetic acid and made isotonic by adding sodium chloride (1 g.).

(*b*) *Filtered B.E.*—Prepared as described previously. After filtering, the solution (160 ml. + 40 ml. water used to wash the filter) was made isotonic by adding sodium chloride (1·4g.)

(*c*) *Penicillin-treated A.C.E.*—Prepared as described for (*a*), but instead of being filtered sterile, the solution (160 ml.) was adjusted to pH 7·2, rendered isotonic and incubated at 37° for 2 hr. with penicillin (1000 units/ml.).

(*d*) *Penicillin-treated B.E.*—Prepared as described for (*b*) and treated with penicillin as described for (*c*).

(*e*) *Heat- and penicillin-treated bacterial suspension.*—The suspension (160 ml.) was heated to 60° for 30 min., adjusted to pH 7·2, rendered isotonic and treated with penicillin as described for (*c*).

To determine whether the bacterial extracts after any of the above treatments were toxic, each preparation was injected intraperitoneally into a single guinea-pig (500 g.), which was kept under observation for 7 days. Table II (Exp. 1 and 2) summarises the results of two experiments, and it is evident that no toxin was demonstrable under these conditions. Preparations (*c*), (*d*) and (*e*) were made to rule out a possible loss of toxin during filtration of (*a*) and (*b*), although this was unlikely since the filters were of sintered glass. As (*c*), (*d*) and (*e*) were not wholly free from *B. anthracis* and other organisms, the appropriate guinea-pigs were given daily injections of oily penicillin (30,000 I.U.) to try to prevent infection; this dose of penicillin was innocuous in control animals. Although this was inadequate in a few instances, it was clear that the unfiltered extracts were as non-toxic as those filtered.

For comparison with these toxicity experiments, guinea-pigs (500 g.) were injected with the untreated live bacteria from infected guinea-pigs in quantities 1/100 and 1/10,000 of those made into the preparations described above. These animals died very rapidly of anthrax infection; the times for death to occur are included in Table II.

TABLE II.—*Non-Toxicity of Bacterial Extracts when Injected Alone and Together with P.E.*

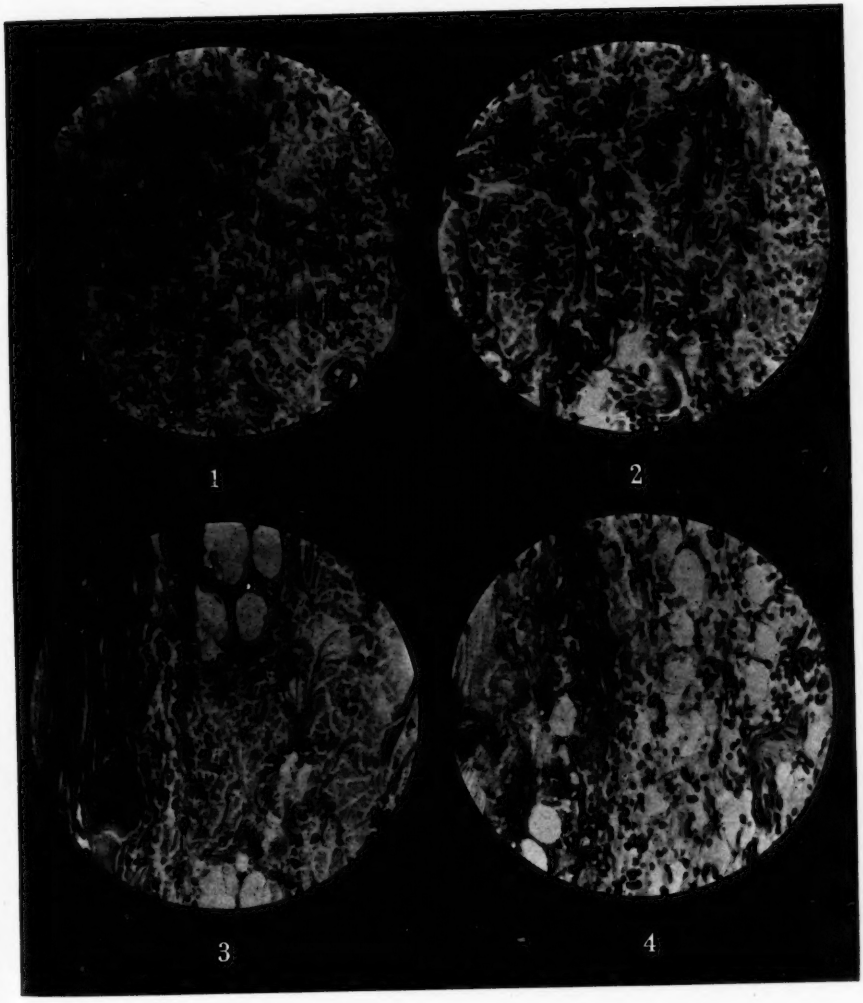
Nature of material injected i/p (160–200 ml.).	Bacterial extracts alone. Exp. 1 (each extract equiv. to $7{\cdot}5 \times 10^{10}$ bacterial chains). No. of guinea-pigs.	Result.	Exp. 2 (each extract equiv. to $10{\cdot}4 \times 10^{10}$ bacterial chains). No. of guinea-pigs.	Result.	Bacterial extracts with solids from P.E. (100 ml.). (each extract equiv. to $9{\cdot}9 \times 10^{10}$ bacterial chains). No. of guinea-pigs.	Result.
Filtered A.C.E. (sterile)	1	Alive	1	Alive	1	Alive
Filtered B.E. (sterile)	2*	„	1	„	1	„
Penicillin-treated A.C.E. (non-sterile)	1	„	1	Died of anthrax 6th day	1	Died of anthrax in 40 hr.
Penicillin-treated B.E. (non-sterile)	1	„	1	Died of anthrax in 48 hr.	1	Alive
Heat- and penicillin-treated extract (non-sterile)	1	„	1	Alive	1	Died of streptococcal infection in 52 hr.
Live bacteria 1/100 quantity in each extract	2	Died in 15 and 16 hr.	3	Died in 10, 11 and 12 hr.	3	Died in 11, 12 and 13 hr.
Live bacteria 1/10,000 quantity in each extract	1	Died in 38 hr.	4	Died in 17, 18, 19 and 19 hr.	4	Died in 18, 19, 20 and 21 hr.

The guinea-pigs used weighed 500 g. and were observed for 7 days.
* The result of a single preliminary experiment with filtered B.E. is included here.

DESCRIPTION OF PLATE.

Tissue changes in guinea-pigs 24 hr. after intradermal injection (0·2 ml.) of solutions (5 per cent w/v) of sterile anthrax products.

FIG. 1.—P.E., adjacent to cutaneous muscle.
FIG. 2.—P.E., a more superficial field.
FIG. 3.—A.C.E.
FIG. 4.—B.E.



Keppie, Smith and Harris-Smith.

Non-toxicity of P.E.

Quantities equivalent to 75, 100, 150 and 200 ml. of undiluted P.E. were injected into guinea-pigs (500 g.). As described previously (Smith *et al.*, 1953*a*), the undiluted P.E. collected had to be mixed with an equal volume of diluent to ensure satisfactory filtration through "Millipore" filters. Since the maximum volume tolerated by a guinea-pig (500 g.) was 200 ml., the 150 ml. and 200 ml. quantities of undiluted P.E. were obtained in isotonic solution by diluting with water before filtration, freeze-drying and reconstituting. Any possible adverse effects of this treatment were ruled out for the 75 ml. and 100 ml. quantities by diluting with Locke's solution and injecting 150 ml. and 200 ml. respectively after filtration.

Table III summarises the results obtained in 3 experiments with different batches of P.E. It includes the results of controls given normal heparinised guinea-pig plasma. No toxic extracellular substances were detected in the P.E. under these conditions.

TABLE III.—*Non-Toxicity of P.E. when Injected Alone.*

Volume of material injected (i.p., ml.).	Control (normal heparinised plasma).		P.E. (combined results on three different batches).	
	No. of guinea-pigs.	Result.*	No. of guinea-pigs.	Result.*
200	5	3 alive, 2 died (1st and 6th day)	4	3 alive, 1 died (6th day).
150	10	Alive	5	Alive
100	3	2 alive, 1 died (7th day)	6	„
75	—	—	2	„

*The animals were observed for 7 days.

Non-toxicity of bacterial extracts and P.E. when injected together.

The experiment which showed the non-toxicity of bacterial extracts when injected alone was repeated but instead of adding sodium chloride to make the preparations isotonic, the freeze-dried solids from 100 ml. undiluted P.E. were dissolved in solutions (*a*), (*b*), (*c*), (*d*) and (*e*) described above. The results of this experiment are included in Table II. The mixtures of products were non-toxic under these conditions.

Tissue-damaging Activity of B. anthracis *Products.*

Watson, Cromartie, Bloom, Heckly, McGhee and Weissman (1947) showed that crude oedema fluid, and a fraction of it, produced a lesion if injected intradermally into rabbits. The histopathology of this lesion was similar to that produced by *B. anthracis* in a similar site. Some tissue damage was produced by our products which, however, was not extensive or of long duration.

Intradermal injections of 5 per cent w/v solutions (0·2 ml.) of A.C.E., B.E. and P.E. (dialysed) were made in guinea-pigs.

Macroscopically A.C.E. and B.E. produced only a trace of oedema, which quick-

ly faded. P.E. produced a small oedematous plaque within 24 hr., but the oedema and congestion were short-lived and were diminishing by 48 hr.

The tissue changes are shown in Fig. 1, 2, 3, and 4. The tissue reactions produced by P.E. and the two bacterial extracts were similar, and differed only in degree, the P.E. reaction being the more widespread. The whole depth of the corium was involved, the changes being greatest in the deeper layers adjacent to the cutaneous muscle. No selective damage to any particular tissue was found.

The site of the injection showed a loss of the superficial layers of the epidermis, leaving an accumulation of disintegrating polymorphs with a fibrinous coagulum. The deeper layers of the epidermis showed little change although only a thin layer of the epithelium remained. The papillary connective tissue in a small area under the bleb showed hyaline necrosis with a few polymorphs at the margin. Downwards through the corium there was some oedema which pushed the collagen fibres apart. Polymorphs were widely scattered in all the spaces, but were accumulated in the clefts between fibres in the mid-zone of the corium (Fig. 2), where a localised abscess may be formed. There was no evidence of destruction or fragmentation of collagen (Watson *et al.*, 1947), but some fibres showed hyaline change and stained deeply with eosin (Fig. 2). Oedema was greater towards the muscle, and there was some fibrinous coagulum in the tissue spaces. Scattered polymorphs were present in the oedematous tissue and some red cells were free in the tissue spaces (Fig. 1). Deep to the muscle there was oedema and fibrinous accumulation, and there the red cells were present in greater numbers and there were haemorrhages from damaged capillaries.

The peak of the reaction had occurred by 24 hr. and by 48 hr. resolution had begun. On the 7th day healing was almost complete.

The Aggressin Activity of B. anthracis *Products.*

A.C.E., B.E. and P.E. have been tested for aggressin, or the capacity to interfere with the host defence mechanisms (Bail, 1904; Bail and Weil, 1911). Tests for such activities as the promotion of infection by sub-lethal doses of organisms and interference with the phagocytic and bactericidal power of blood will be described. The two latter tests have been developed into biological assays which can be used for detecting aggressin during the chemical purification of crude products. The activity of crude products in the tests will be described here, but the results of the chemical fractionation and a statistical examination of the assays will be given elsewhere.

The promotion of B. anthracis *infection in guinea pigs by* B. anthracis *products.*

Preliminary experiments showed that 50 *B. anthracis* spores in gelatin-Locke solution (20 ml.) were non-lethal for guinea-pigs (300–350 g.) by the intraperitoneal route; 1×10^4 spores by this route are needed to kill all animals. In order to test solutions of P.E. (2 per cent w/v), A.C.E. (1·25 per cent w/v) and B.E. (1·25 per cent w/v) for their capacity to promote infection, 50 spores were injected with 20 ml. of each of these solutions using groups of 5 guinea-pigs. All of these animals died, whereas no deaths occurred in 30 control animals.

Aggressin activity was thus demonstrated in the intracellular products as well as in the extracellular products. The large amount of material necessary for this *in vivo* test makes it unsuitable as a routine assay for chemical fractionation.

The antiphagocytic activity of B. anthracis *products.*

The A.C.E., B.E., and P.E. were examined for antiphagocytic activity in the following *in vitro* phagocytosis test. Citrate has a definite antiphagocytic action in this test (Allgöwer, 1947): therefore the samples of P.E. were dialysed against distilled water before use.

To fresh defibrinated guinea-pig blood (0·4 ml.) contained in a waxed bottle (5 ml.) gelatin-Locke solution (0·4 ml.) containing the test material was added, and the mixture was kept at 37° for 30 min. A broth suspension (0·2 ml.) of *B. anthracis* was then added. This suspension contained ten times as many organisms in 0·2 ml. as there were polymorphs in 0·4 ml. of the blood. It was prepared by adding the correct number of spores to broth and keeping the mixture at 37° for 30 min. The spores had then formed small stainable diploid organisms which were easy to count when inside the phagocytes. The final mixture was rotated at 37° for 30 min., cooled in ice and centrifuged at 500 r.p.m. (10 min.) and then at 2000 r.p.m. (10 min). The well-defined white cell layer was filmed and stained with Leishman's stain. Organisms in 100 phagocytes were counted, and the mean phagocytic index for the experimental sample was compared with that of the control. Several concentrations of the materials under test were examined, and Table IV summarises the results of one of a number of tests carried out on each sample. The antiphagocytic activity of A.C.E., B.E. as well as P.E. is well marked. Absence of citrate (< 0·3 per cent) in the freeze-dried products was confirmed by the chemical method of Hargreaves, Abrahams and Vickery (1951).

TABLE IV.—*Antiphagocytic and Antibactericidal Activity of* B. anthracis *Products.*

Nature of test material.	Concentration per cent.	Antiphagocytic test. Mean phagocytic index from count of 100 phagocytes.	Antibactericidal test. (Figures are the viable counts in equivalent samples of the mixtures.) Initial	Final.
A.C.E.	*Nil* (control)	6·13	104	23
	5	0·8	103	48
	2·5	4·29	100	55
	1·25	5·89	96	45
	0·62	—	94	42
B.E.	*Nil* (control)	6·13	85	28
	5	1·32	81	65
	2·5	5·08	89	50
	1·25	4·78	80	41
	0·62	—	88	39
P.E. (Batch 1)	*Nil* (control)	9·21	91	25
	5	7·62	91	57
	2·5	9·59*	95	43
	1·25	—	92	43
	0·62	—	100	33
	0·31	—	90	29
(P.E. Batch 2)	*Nil* (control)	10·7	91	25
	5	5·32	90	90
	2·5	7·40	87	67
	1·25	10·2*	85	52
	0·62	9·93*	91	48
	0·31	—	96	41

Whole defibrinated guinea-pig blood was used. Details of the tests are given in the text.
* Figure not significantly lower than the control.

The antibactericidal activity of B. anthracis *products.*

This test was carried out as described for the anti-phagocytic test, except that the number of germinated *B. anthracis* spores added was one-tenth of the number of polymorphs in the

blood, and rotation of the final mixture was continued for 1½ hr. Immediately after adding the organisms and before rotation at 37°, a sample was taken from each waxed bottle. After appropriate dilution in gelatin-Locke, a measured volume was spread on 4 nutrient agar plates for a viable count. The same procedure was repeated after rotation in the hot room, but 6 plates were used for each sample The bactericidal action of defibrinated guinea-pig blood, which reduced the viable count of the control to a low figure, was inhibited by A.C.E., B.E. and the two citrate-free samples of P.E.; the greater the aggressin activity of the test material, the higher was the final viable count.

Table IV includes typical results which demonstrate anti-bactericidal activity in A.C.E. and B.E. as well as in P.E.

The nature of the bactericidal action of defibrinated guinea-pig blood.

It could be shown that normal guinea-pig serum had no bactericidal action on broth cultures of *B. anthracis*. The presence of an anthracidal substance in leucocytes (Bloom, Watson, Cromartie and Freed, 1947) suggested the possibility that secretion of such a substance by the white cells might occur after the bacilli were added (see also Bail and Weil, 1911). This was disproved by determining the fate of all the organisms used in phagocytosis tests. These were carried out without the addition of any experimental material. After 30 min. rotation at 37°, samples were taken for viable and phagocytic counts as described before. Rotation was continued and further samples were taken at 60 min. and 90 min. Table V gives the results obtained in several experiments. These show that the sum of the organisms remaining viable and the number phagocytosed equalled the number of organisms initially added. This means that under these conditions there is no extracellular bactericidal activity.

TABLE V.—*Nature of the Action of Defibrinated Guinea-Pig Blood on* B. anthracis *from Broth Culture.*

Exp.	Number of organisms added to the system.	Distribution of bacteria after incubation for the time interval stated. 30 min. Viable	Phago-cytosed	=Total	60 min. Viable	Phago-cytosed	=Total	90 min. Viable	Phago-cytosed	=Total
1	6·0	—	—	—	3·7	2·5	6·2	3·8	2·5	6·3
2	9·2	4·9	3·8	8·7	3·6	5·7	9·3	3·3	6·1	9·4
3	9·9	7·0	2·9	9·9	4·9	5·0	9·9	3·5	5·6	9·1
4	2·3	1·3	0·7	2·0	—	—	—	1·1	0·9	2·0

Figures are the total count ($\times 10^6$) in the mixture (1 ml.).
The ratio of organisms to phagocytes in this mixture is approximately 10 : 1.

It is possible that the viable counts after the action of the blood were reduced by the number of polymorphs present, since these may each have contained one viable organism which gave rise to a colony in the viable count. Application of this correction does not affect the main conclusion, that there is no extensive extra-cellular bactericidal activity.

These results were confirmed in a smaller number of experiments using the conditions of the bactericidal test.

The Immunising Activity of B. anthracis *Products.*

The literature already reviewed (Smith *et al.*, 1953*b*) clearly shows that extracellular products of *B. anthracis* whether produced *in vivo* or by special conditions *in vitro* contain a protective antigen. Attempts to detect such an antigen in extracts of bacteria grown *in vitro* have been unsuccessful, but preparations from bacteria grown *in vivo* have not so far been examined. The results of active immunisation experiments on A.C.E. and B.E. are therefore of special interest.

P.E., A.C.E. and B.E. were examined for their power to produce active immunity in the following test. Guinea-pigs (300–350 g.) were given 3 subcutaneous injections (1 ml.) of the test material at 7-day intervals. One week after the last injection the animals were challenged with 1000 L.D. of *B. anthracis* spores. Deaths were recorded up to 10 days. This test is now used as a biological assay of the protective antigen in the chemical fractionation of P.E. Full details of this fractionation and of the biological assay, especially the statistical treatment of data, will be published elsewhere.

Activity of P.E.

Several batches of freeze-dried P.E. have been tested for their immunising power in guinea-pigs with similar results. The combined results show that with concentrations (per cent) of 4, 1, 0·5 and 0·025 the number of survivors was 10/10 35/38, 11/15 and 3/15 respectively. It must be noted that a 4 per cent solution of freeze-dried P.E. is approximately equivalent to undiluted P.E. collected from guinea-pigs ; approximately 1/3rd of the dry solids are the sodium citrate and salts of the Locke's solution added during harvesting.

Further experiments have shown that P.E. does not lose appreciable immunising activity on freeze-drying and does not confer passive immunity ; it also protects rabbits against a *B. anthracis* infection. When examined separately, both the blood plasma and the cavity exudate from infected guinea-pigs have immunising activity.

Inactivity of A.C.E. and B.E.

The following solutions, 3 per cent w/v A.C.E., 2·5 per cent w/v A.C.E. (different batch) and 3 per cent w/v B.E. gave no protection to 5, 4 and 4 guinea-pigs respectively in the test for protective antigen. The quantity of bacterial extract used for one guinea-pig in these experiments was derived from the bacteria removed from approximately 100 ml. of P.E. in the harvesting procedure (Smith *et al.*, 1953*a*). This quantity of P.E. by virtue of its content of extracellular antigen would immunise 150 guinea-pigs. Tests have shown that treatment with ammonium carbonate or shaking with ballotini does not affect the protective activity of P.E.

The results obtained with these bacterial extracts show that the intracellular products of *B. anthracis* grown *in vivo* are as devoid of immunising activity as are similar *in vitro* preparations. This further confirms the essentially extracellular nature of the immunising antigen of *B. anthracis.*

There was a possibility that the immunising antigen was in high concentration at the surface of the cell, and was removed by the Locke's solution when the bacteria were washed. This was excluded by examination of the activity of the

three individual wash solutions. Activity was only apparent in the first washing, and this was no more than that accounted for by the presence of P.E.

Connection between the protective antigen and the aggressin of P.E.

The fractions of P.E. resulting from purification studies have been tested in parallel for protective antigen and for aggressin by means of the tests described earlier. In samples from three different types of fractionation, preliminary results have shown an interesting correlation worth mentioning here between protective antigen and aggressin. The results are summarised in Table VI.

TABLE VI.—*Relative Activity of P.E. Fractions in Tests for Protective Antigen and Aggressins.*

Type of fractionation.	Fraction.	Lowest active concentration* (per cent)		
		Immunising test.	Antiphagocytic test.	Antibactericidal test.
Cohn/Edsall plasma fractionation	1	0·2	0·17	0·17
	2	0·2	0·5	0·5
	3	Inactive at 1·6	Inactive at 1·5	Inactive at 1·5
Ammonium sulphate	1	0·2	0·17	0·5
	2	0·8	0·5	1·5
	3	Inactive at 1·6	Inactive at 4·5	Inactive at 4·5
A combination of barium acetate and ethanol	1	0·2	No test	0·26
	2	0·05	,,	0·06
	3	1·6	,,	1·6

* The volume of solutions used and other details of these tests are as described in the text ; figures in the table are concentrations of solutions used as test material.

The samples tested were not pure materials, and therefore the possibility remains that two different substances are responsible for each activity. However, the correlation up to the present is noteworthy, and is supported by the finding that a sample of purified protective antigen produced in artificial cultures (Strange and Belton, 1953 ; personal communication) had aggressin activity in both tests.

DISCUSSION.

The evidence given in the first part of this paper strongly indicates the absence of any well-defined endo- or exotoxin in the materials from a *B. anthracis* infection in guinea-pigs. This supports earlier work (Eurich and Hewlett, 1930 ; Sobernheim, 1931 ; King and Stein, 1950) using materials prepared by culture *in vitro*. Gross toxicity is almost certainly ruled out by our experiments. The quantities of materials injected into a single guinea-pig were unusually large, and the methods of preparing the extracts were very mild. An absence of gross toxicity might be expected in view of the large numbers of bacilli present in the guinea-pig at death, which suggests that the toxicity per organism cannot be large, or the host would probably die before so many could be produced.

The important inference to be drawn from these toxicity experiments is that the killing power of *B. anthracis* is associated mainly with the growth of the organism in the tissues of the host, rather than with any particularly active endo-

or exotoxin. The rapid death produced by the relatively small initial dose of live organisms described in the toxicity experiments shows the importance of this growth. There are two essentials for rapid growth in the host tissues, a satisfactory nutritional medium (Bacon, Burrows and Yates, 1951), and the production by the organisms of substances which combat the normal host defence mechanisms. At the moment, therefore, it is thought that the most important chemical substances to study in connection with the virulence of *B. anthracis* in guinea-pigs are the aggressins. They are present in the intra- and extracellular products of the organism, and work is in progress on the fractionation of these crude materials for the compounds responsible for activity. It is relevant to mention here that Sterne (1937), on the basis of observations on the virulence and immunising power of *B. anthracis* variants, suggested that virulence was perhaps determined by two factors. One was connected with the capsule of the organism, but the other was extracellular and was also responsible for immunising power. The results described above support this theory Both intra- and extracellular aggressins are present and evidence from chemical fractionation connects an extracellular aggressin with the protective antigen.

Once the organisms can grow abundantly, the death of the host could result either from the combined action of a number of relatively weak or slowly acting harmful effects, or through the host's tissues being deprived of essential nutrients by the metabolic activity of the organism.

A number of the effects of infection mentioned in the literature (some of which have been noticed in this work with guinea-pigs) could be harmful. A slight tissue-damaging factor has been demonstrated in this and other work (Sobernheim, 1931 ; Watson *et al.*, 1947). The abnormalities of the blood in the terminal phases such as the slight haemolysis, the increased red cell fragility, the delayed clotting and the agglutination of red cells must result from the action of some as yet undefined harmful product of *B. anthracis*. The possibility that calcium metabolism is interfered with has been suggested by Bloom, McGhee, Cromartie and Watson (1947), and de Moulin (1936) has drawn attention to evidence of damage to the tissues of the nervous system. In this connection it is important to bear in mind that weakly toxic materials—*e.g.*, the lecithinase of McGaughey and Chu (1948)—may be fixed on the host tissues as soon as they are formed, and would not be detected in the products harvested.

It should be feasible in connection with the second of these possibilities to determine whether the bacilli deprive the host of essential materials by studying the rate at which these organisms remove physiological essentials from the fluids of the host.

In connection with anthrax in the guinea-pig, it is important to decide whether the final septicaemia, which appears to coincide with the period of greatest distress in the animal, is essential to cause death. In view of the reports in the literature (Cromartie, Bloom and Watson, 1947 ; Bloom, McGhee, Cromartie and Watson, 1947) that death from anthrax occurs in some species without a marked septicaemia, blood films were examined from approximately 200 guinea-pigs dying from the form of experimental anthrax used in this work. In every case the blood contained large numbers of bacilli. The counts made on the blood of the six animals described earlier in this paper are additional evidence that a marked septicaemia precedes death in the guinea-pig. This is supported by the fact that the total yield of bacteria collected from successive batches of 100 guinea-pigs (Smith *et al.*,

1953*a*) is a remarkably constant amount. Further work is necessary to examine the suggestion that guinea-pigs treated differently from those under discussion may die from anthrax in the absence of a marked septicaemia.

Study of the virulence of *B. anthracis* in guinea-pigs is being continued on the lines indicated in this discussion.

SUMMARY.

As a preliminary to chemical fractionation, products of *B. anthracis* harvested from infected guinea-pigs have been examined in various biological tests connected with virulence.

Large quantities of intra- and extracellular products were non-toxic when injected intraperitoneally.

Intra- and extracellular products contain aggressins as shown by their ability to promote infection by sub-lethal doses of *B. anthracis* and to interfere with phagocytosis.

Extracellular products produced active immunity to anthrax in guinea-pigs but intracellular products did not. Evidence is presented that an extracellular aggressin and the protective antigen may be the same substance.

We are indebted to Dr. Joan Ross for the histological report and for the photographs. Our thanks are also due to Mr. T. Snape for excellent technical assistance. Acknowledgement is made to the Chief Scientist, Ministry of Supply, for permission to publish this paper.

REFERENCES.

ALLGÖWER, M.—(1947) *Schweiz. med. Wschr.*, **77**, 40.
BACON, G. A., BURROWS, T. W., AND YATES, M.—(1951) *Brit. J. exp. Path.*, **32**, 85.
BAIL, O.—(1904) *Zbl. Bakt., Abt. I*, **47**, 270.
Idem AND WEIL, E.—(1911) *Arch. Hyg., Berl.*, **73**, 218.
BLOOM, W. L., WATSON, D. W., CROMARTIE, W. J., AND FREED, M.—(1947) *J. infect. Dis.*, **80**, 41.
Idem, MCGHEE, W. J., CROMARTIE, W. J., AND WATSON, D. W.—(1947) *Ibid.*, **80**, 137.
CROMARTIE, W. J., BLOOM, W. L., AND WATSON, D. W.—(1947) *Ibid.*, **80**, 1.
DE MOULIN, F. W. K.—(1936) *Ned-ind. Bl. Diergeneesk.*, **48**, 126.,
EURICH, F. W., AND HEWLETT, R. T.—(1930) 'A System of Bacteriology.' London (Medical Research Council), **5**, 439.
GLADSTONE, G. P.—(1946) *Brit. J. exp. Path.*, **27**, 394. (1948) *Ibid.*, **29**, 379.
HARGREAVES, C. A., ABRAHAMS, H. M. D., AND VICKERY, H. B.—(1951) *Analyt. Chem.* **23**, 469.
KING, H. K., AND STEIN, J. H.—(1950) *J. gen. Microbiol.*, **4**, 48.
MCGAUGHEY, C. A., AND CHU, H. P.—(1948) *Ibid.*, **2**, 334.
SMITH, H., KEPPIE, J., AND STANLEY, J. L.—(1953*a*) *Brit. J. exp. Path.*, **34**, 471. (1953*b*) *Ibid.*, **34**, 477.
SOBERNHEIM, G.—(1931) 'Handbuch der Pathogenen Mikroorganismen.' Jena (Gustav Fischer), **3**, 1041.
STERNE, M.—(1937) *Onderstepoort J. vet. Sci. and Animal Ind.*, **8**, 271.
TREFFERS, H. P.—(1947) *Annu. Rev. Microbiol.*, **1**, 263.
WATSON, D. W., CROMARTIE, W. J., BLOOM, W. L., HECKLY, R. J., MCGHEE, W. J., AND WEISSMAN, N.—(1947) *J. infect. Dis.*, **80**, 121.

THE ACTION OF AUREOMYCIN IN PNEUMOCOCCAL INFECTIONS IN NORMAL AND LEUCOPENIC RABBITS AND IN MICE.

J. BOWDEN.

From the Sir William Dunn School of Pathology, Oxford.

Received for publication April 30, 1953.

PENICILLIN and terramycin can eliminate pneumococcal infection in rabbits made profoundly leucopenic with injections of benzene (Gowans, 1953). It was concluded that these drugs are strongly bactericidal *in vivo* and are effective in the absence of the normal body defences. In the present experiments the action of aureomycin on pneumococcal infection in leucopenic rabbits was investigated. This is of some interest, since there is evidence that *in vivo* its activity is greater than *in vitro* tests would suggest (*e.g.*, Klein, Schorr, Tashman and Hunt, 1950). The action of aureomycin on pneumococcal infection in normal mice was also examined.

The Action of Aureomycin on Pneumococcal Infection in Normal and Leucopenic Rabbits.

The methods were similar to those used by Gowans (1953). Normal rabbits and rabbits whose circulating leucocytes had been reduced to below 250 cells/c.mm. by repeated injections of benzene (Selling, 1911; Rich and McKee, 1934) were infected intradermally on both flanks with virulent Type 1 pneumococcus (Wellcome CN33 strain). Treatment with aureomycin (50 mg./kg. body wt. 6-hourly) was started 6 hours later, a double dose being given for the first injection. The commercial preparation of aureomycin hydrochloride buffered with sodium glycinate was given intravenously as a 1 per cent solution in saline. The rabbits were killed 12 hours after the last dose of antibiotic; the skin lesion on one side was excised, homogenized and cultivated in broth, while that on the other was examined histologically. Cultures of blood and oedema fluid were made during the experiments. All material for cultivation was highly diluted, and incubation was for 72 hours.

RESULTS.

Descriptions of the pneumococcal skin lesions in untreated normal and leucopenic rabbits have been given by Gowans (1953) and were confirmed in the present work.

Normal rabbits treated with aureomycin.

Four normal rabbits were treated with aureomycin. Three of these were killed 18, 24 and 48 hours after infection respectively; the fourth was treated for 48 hours and allowed to survive.

Macroscopically the redness and oedema were much less marked than in the corresponding untreated rabbits, and skin purpura was never seen. The lesions reached a maximum size of about 4–5 cm. between 12 and 18 hours after infection and thereafter regressed until at the end of 48 hours they were scarcely visible.

Absorption of the oedema fluid occurred more slowly, and some was still present in the abdominal wall 4 days after the end of the experiment.

Histologically the skin infection became localized and the number of bacteria declined progressively. At 18 hours the massive cellular infiltration consisted almost entirely of granulocytes and, as in the untreated rabbits, these frequently contained cocci in the denser parts of the lesion. However, there was no more phagocytosis by granulocytes in the treated than in the untreated animals and the vast majority of cocci remained extracellular. By 24 hours there were many macrophages, and many of these contained the Gram-positive remains of cocci. At 48 hours the tissues appeared to be completely free of organisms and were sterile on culture ; after 24 hours they still contained many viable cocci. Blood cultures, positive at first, became persistently negative after 18 hours.

Leucopenic rabbits treated with aureomycin.

This group consisted of four rabbits with leucocyte counts persisting below 250 cells/c.mm. throughout the experiments. Two were treated for 24 hours and two for 48 hours.

Macroscopically the lesions were paler than in the untreated leucopenic animals; oedema was very slight and no purpura developed.

Histologically the lesions always contained a few granulocytes, but the numbers rarely exceeded 5 per high-power field and they showed no phagocytosis. There were a number of scattered macrophages, a few of which contained many Gram-positive cocci. During treatment the number of visible cocci declined rather more slowly than in the normal animals, and even after 48 hours one or two morphologically unaltered Gram-positive organisms still remained. At 24 hours these apparently normal cocci were plentiful, and could be seen both diffusely scattered throughout the tissues and often gathered into small foci near blood-vessels ; by 48 hours, however, they were confined almost exclusively to the latter situation. A few swollen normally staining forms were seen, and in the parts of the sections where the bacteria had been most numerous, there were patches of fine granular Gram-negative débris often showing diplococcal form.

Blood cultures, positive at first, became negative after 24 hours and remained so until death. Cultures of the skin lesions and oedema fluid were sterile at 48 hours, though not at 24 hours.

The Action of Aureomycin on Pneumococcal Infection in Normal Mice.

Thirty female white mice weighing about 20 g. were infected intraperitoneally with 0·2 ml. of a 1/10,000 dilution of a 5-hour serum-broth culture of Type 1 pneumococcus ; this inoculum contained about 3500 organisms. Virulence was maintained by mouse passage and a lethal dose was always less than 50 organisms. Aureomycin hydrochloride was given subcutaneously, a 0·5 per cent unbuffered solution in normal saline with an initial dose of 75 mg./kg. body wt. 12 hours after infection, followed by two further doses of 50 mg./kg. at 4-hourly intervals. This was always found to cure the infection. The mice were killed at various times after infection, and a cotton-wool swab was introduced into the peritoneal cavity and then smeared on blood agar. To ensure that any failure of growth was not due to the persistence of an inhibitory concentration of aureomycin the swab was then dropped into a tube containing 20 ml. of serum broth, incubated and subsequently plated out. All plates were incubated for 48 hours. Films of the peritoneal exudate were made by drawing slides over the surface of the viscera, drying in air, heat-fixing and staining with Giemsa's and Gram's stain.

RESULTS.

Untreated mice died 22–36 hours after infection. As the infection progressed the exudate became crowded with granulocytes and with extracellular pneumococci. There was almost no phagocytosis by granulocytes, but careful searching of the films showed a few macrophages, some of which contained up to 60 organisms, morphologically unaltered and Gram-positive. These cells were not observed after 14 hours.

In the treated mice the number of extracellular cocci declined rapidly during the first 4 hours of treatment and then more slowly over the next 6 hours. At this time no pneumococci were seen in the films, but cultures remained positive for a further 2 hours. Swollen deeply stained cocci were numerous throughout treatment, and after 2 hours large numbers of extracellular organisms were seen in various stages of disintegration. These ranged from morphologically normal Gram-negative diplococci to an amorphous mass of Gram-negative débris. During the first 8 hours of treatment the inflammatory response was intense and consisted almost entirely of granulocytes. By 10 hours a number of macrophages had appeared. Phagocytosis by these cells, however, was negligible.

DISCUSSION.

Normal and leucopenic rabbits were infected with virulent pneumococci and treated intravenously with large doses of aureomycin. In the leucopenic animals the bacteria disappeared from the lesions more slowly and the cultures remained positive longer than in the normal animals. The difference was not great, however, and in both groups the lesions were sterile in 48 hours. There was no more phagocytosis by granulocytes in normal rabbits treated with aureomycin than in the untreated controls. These experiments show that the powerful bactericidal action of aureomycin against pneumococcal infection in rabbits does not depend on the activity of granulocytes.

The cocci in the skin of the leucopenic rabbits became reduced to finely granular Gram-negative débris often showing diplococcal structure. It was concluded that these organisms had undergone lysis.

The experiments with mice confirmed the view that during treatment with large doses of aureomycin phagocytosis by granulocytes plays no part in eliminating pneumococci and that the organisms undergo extracellular lysis.

In all the lesions of the leucopenic rabbits there were a number of macrophages. Most of them showed no phagocytosis, but in the few which did the number of ingested cocci was considerable. However, it is thought unlikely that phagocytosis by so few cells could have assisted materially in eliminating the infection, especially as much extracellular bacterial debris was always present in sterile lesions. In the lesions of normal treated rabbits many macrophages containing the remains of pneumococci were seen in the later stages, but whether they were removing dead cocci or had actually contributed to the killing of the organisms is not known. Phagocytosis of pneumococci by macrophages was also seen in peritoneal exudate from mice, but again the number of cells involved makes it unlikely that their contribution to the killing of the organisms was important.

It is concluded from these experiments that large systemic doses of aureomycin exert a rapid and complete bactericidal action on pneumococci in rabbits and mice.

This work was suggested by and carried out under the supervision of Professor Sir Howard Florey, to whom I am greatly indebted for advice and encouragement. I should also like to acknowledge the invaluable technical assistance given by Mr. I. Chinn, Mr. D. W. Jerrome, Mr. A. Graham and Mr. N. Smith. The work was carried out during the tenure of a Medical Research Council "Studentship for Training in Research Methods."

Aureomycin was supplied through the courtesy of Lederle Laboratories Division, American Cyanamid Company.

REFERENCES.

GOWANS, J. L.—(1953) *Brit. J. exp. Path.*, **34**, 35.
KLEIN, M., SCHORR, S. E., TASHMAN, S., AND HUNT, A. D.—(1950) *J. Bact.*, **60**, 159.
RICH, A. R., AND MCKEE, C. M.—(1934) *Johns Hopk. Hosp. Bull.*, **54**, 277.
SELLING, L.—(1911) *Beitr. Path. Anat.*, **51**, 576.

SERUM ANTIBODIES IN MUMPS

B. GHOSH RAY AND R. H. A. SWAIN.

From the Bacteriology Department, University of Edinburgh.

Received for publication May 13, 1953.

MUMPS is a generalised infection in which the parotid glands are very frequently though not invariably involved. Other organs may be affected and Banks (1951) states that complications of the disease may be meningo-encephalitis in 10 per cent of cases, orchitis in up to 37·5 per cent, pancreatitis in 7 per cent and oophoritis less frequently.

In the past these complications were recognised as a part of the disease of mumps only when they were accompanied by obvious swelling of the parotid glands. Since the development of serological methods during the last ten years it has been possible to decide with certainty that these complications can occur without any clinical sign of involvement of the salivary glands. Kilham (1949) found that in 25 proved cases of mumps meningo-encephalitis 13 had no glandular enlargement. Henderson (1952) while investigating an outbreak of mumps in a boarding school found that of 11 cases of mumps meningitis 4 had no parotitis.

The clinical features of mumps meningo-encephalitis are those of a mild meningeal reaction accompanied by a lymphocytosis in the cerebrospinal fluid and followed invariably and rapidly by complete recovery. The resemblance of this illness to non-paralytic poliomyelitis and to the various imperfectly understood infections known as " aseptic meningitis " is so close that the diseases may be quite indistinguishable on clinical grounds alone.

Benign meningitis is common among children in this country during the summer and autumn months when both poliomyelitis and mumps epidemics may occur simultaneously. There is thus the need for a practical serological test with which to distinguish mumps meningo-encephalitis from the other types of lymphocytic meningitis.

There has not been universal approval of the serological techniques that are in use. Henle, Harris and Henle (1948) in an analysis of more than two thousand sera showed that the antibody response to the two complement-fixing antigens of the mumps virus followed a regular pattern. Antibodies to the soluble or " S " complement-fixing antigen appeared very early in the disease often reaching a detectable level as soon as the second or third day while antibodies to the virus-bound or " V " antigen were only found after the eighth day of the disease. These findings were not, however, supported by the work of either Aikawa and Meiklejohn (1949) or of Feller and Jordan (1950) who were unable to confirm that the sera taken in the early stages of the disease reacted regularly with the S antigen. These workers used a different strain of the mumps virus and slightly different techniques in the preparation and standardisation of antigens; both are factors which might explain the discrepancy between their results with those of the Henles.

The haemagglutination inhibition test has proved reliable and consistent in the hands of Robbins, Kilham, Levens and Enders (1949), but Lundbäck (1949) was able only to detect a significant rise in titre in 57 per cent of cases by this method.

The purpose of this work was to investigate the reliability of these methods and to compare their specificity with that of the neutralisation test. The use of these techniques for two and a half years has provided an opportunity for their standardisation and has resulted in the disappearance of the conflict of results which was seen in work of the authors quoted above.

MATERIALS AND METHODS.

Sera.

Twenty-five cases of typical mumps with either unilateral or bilateral parotitis were selected for serological examination by the medical staff of the City Fever Hospital in Edinburgh. In addition two children in the family of one of us (R. H. A. S.) were investigated thoroughly during and after typical attacks of mumps. A laboratory worker provided samples of serum at intervals for over a year following her recovery from the disease. Three cases of meningo-encephalitis in patients recently exposed to mumps and without any sign of parotitis were also investigated and are included in the series which thus comprises a total of 31 patients.

As a sample of the healthy adult population sera were obtained from 54 fourth year medical students whose average age was 22 years. In 32 students it was possible to obtain a definite history of infection in the past. Further sera which were examined included 38 from voluntary blood donors and 12 from the cord blood of new born infants.

Serum samples were obtained as early as possible after the onset of mumps and again after an interval of at least 5 or, if possible, 10 days. Sera were transferred at the first opportunity to a dry ice cabinet where they were preserved at − 35° until required. They were inactivated at 56° for 30 min. before use in complement fixation and haemagglutination inhibition tests but were not so treated for the neutralisation tests. The practice of re-inactivation of the sera for 5 min. at 56° immediately before the preparation of serum dilutions was useful in overcoming any anticomplementary activity and was adopted as a routine.

Viruses.

The Enders strain of the mumps virus was obtained from the National Institute for Medical Research, Mill Hill, and was used for the preparation of all the antigens used in the standard serological tests. Two other strains which were isolated in Edinburgh were used for some comparative haemagglutination inhibition studies.

The " James " strain was isolated in 1950 from the saliva of a hospital patient ; it was readily adapted from the amniotic cavity to the allantoic cavity of the chick embryo where it was cultivated as a routine. Both these strains were propagated by inoculation by the allantoic route in 8-day-old chick embryos which were then incubated at 35° for 5 days.

The " Paul " strain was isolated in 1951 from a child who had never been in hospital and who had had no contact with any of the hospital patients in this series. This strain could only be cultivated in the amniotic cavity of the chick embryo and after 24 amniotic passes it has not been possible yet to adapt it to grow in the allantoic cavity.

Both these Edinburgh strains were identified by their specific reaction in complement fixation and haemagglutination inhibition systems with a rabbit antiserum prepared against the Enders strain. The two strains also produced antibodies in the rabbit which reacted with the Enders strain of the virus. Finally the sera from the patients themselves reacted specifically with their homologous virus as well as with the Enders virus.

Complement fixation tests.

The technique of Enders and Levens (1948) was used without modification. Complement was provided by guinea-pig serum, preserved at − 35°, and titrated on the day of the test ; 2 M.H.D. were added to each tube.

Haemolytic system.—Sheep's red blood cells (1 per cent) sensitised with 1 M.H.D. of

amboceptor were used. After titration of the complement the cells were stored at 4° overnight and were reincubated on the following morning at 37° for 15 min. before use.

Antigens.—Two antigens were used. The first a viral (V) antigen was a pool of allantoic fluid of high haemagglutination titre, dialysed against sterile phosphate buffer of pH 7·2 and clarified by centrifuging at low speed; the second was a soluble (S) antigen prepared by a method which was a modification of that of Henle *et al.* (1948).

To prepare the S antigen whole allantoic membranes of infected chick embryos were dried between the folds of sterile filter paper and a 20 per cent w/v suspension was made by grinding in Ten Broeck tubes with normal saline. This suspension was cleared of coarse particles by low speed centrifugation and was then spun at 15,000 r.p.m. for 1½ hr. The resulting supernatant was used as the S antigen; it had little or no haemagglutinating activity and was devoid of virus elementary bodies when examined by electron microscopy.

Both antigens were standardised against the same rabbit antiserum and were used in the test at a dilution which was one-half of that determined as the end point in a titration of the box type. Control antigens were prepared according to these methods from allantoic fluid and allantoic membranes taken from normal embryos of the same age as those supplying the mumps antigens.

Final readings in the tests were made after exactly 30 min. incubation in the 37° water bath. The end point of any titration was taken as the last tube to show 50 per cent haemolysis or less, and the results are expressed in terms of the initial dilution of the serum. No readings by interpolation were made.

Haemagglutination Inhibition (H.I.) Tests.

These were performed by the method of Robbins *et al.*, (1949) in which 4 haemagglutinating doses (8 units/ml.) of the virus are added to each tube and the virus serum mixtures are held at 37° in the water bath for 1 hr. before adding the red blood cells. The fowl erythrocytes used were obtained by wing vein puncture from six brown Leghorn cockerels which were reserved for the purpose. The results are expressed in terms of the final dilution of the serum.

Neutralisation Tests.

These were done by a modification of the technique of Leymaster and Ward (1948). A virus suspension of constant infectivity was provided by a pool of allantoic fluid preserved at −76°; the same pool was used for all the neutralisation tests. The infectivity titre of this pool was estimated before and after the tests were completed; no significant loss occurred and the difference in end point was within experimental error (log. 0·35). To each serum were added 100 infective doses (I.D. 50) of the virus dilution, and the mixtures after standing 1 hr. at room temperature were used to inoculate groups of 8-day old chick embryos. After incubation at 35° for 5 days the fluids were harvested and tested for the presence of virus by haemagglutination. In some cases virus-serum mixtures were inoculated into groups of three eggs; in others six were used. The neutralising titre of the serum was expressed as the reciprocal of the final dilution of the serum which prevented the infection of 50 per cent or more of the embryos.

Batches of serological tests of all kinds were so arranged that a series of samples of serum from a single patient was invariably examined simultaneously. In order to check the experimental error of the methods fifteeen replicate titrations were set up by the complement fixation and by the haemagglutination inhibition methods. Using the same sera and reagents and performing the tests on one day, the maximum variation of a 2-fold (1 tube) difference was observed in 25 per cent of the titrations performed. Thus 4-fold differences in serum titrations were significant.

RESULTS.

The results of tests on 66 sera from 31 cases of mumps are shown in the graph (Fig. 1) where antibody levels obtained by different serological methods have been consolidated and are plotted in terms of negative logarithmic values.

The neutralising antibody rose sharply on the 5th-6th day of the disease from an initial titre of approximately 16 to reach an average maximum level of

650 by the 13th–14th day; in every case at least an 8-fold rise in titre was recorded. A high level was maintained during the next 2–3 months and thereafter the amount of the antibody slowly diminished. After 18 months however the level was still elevated and persisted at a figure four times higher than that determined in the acute stages of the illness.

Although, during the first few days of the disease, the haemagglutination-inhibiting (H.I.) antibody titre had a slight tendency to rise from its initial level of 32 or 64 the main increase did not occur until the 8th–12th day when titres of 1024 and 2048 were rapidly reached. Provided the first of a pair of sera was obtained during the initial 5 days of the disease it was always possible to demonstrate an 8-fold or greater rise in titre. The antibody level was maintained

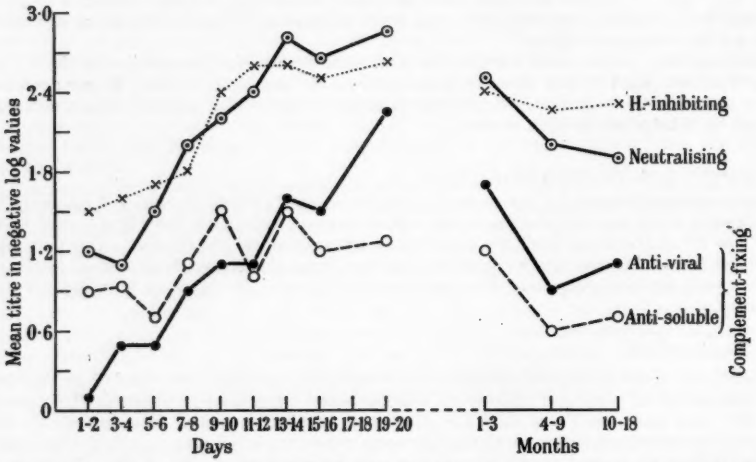


FIG. 1.— The mean logarithmic value of antibody titres in 31 cases of mumps estimated by haemagglutination inhibition, neutralisation and complement fixation during and after the infection.

above 1 in 400 for some 4 weeks when a gradual decline ensued. After 18 months, like the neutralising antibody the H.I. antibody remained considerably above the initial level.

The anti-soluble (anti-S) complement-fixing antibody appeared very early in the disease and titres as high as 16 have been observed on the second day of the disease. The maximum level of 32 or rarely 64 was reached at the 9th–16th day to be followed quickly by a decline which began on the 17th–21st day. Unlike the other antibody levels here described that of the anti-S antibody decreased relatively rapidly in amount and at the end of nine months was often no longer detected. High anti-S titres were not as a rule observed and it was possible to demonstrate a 4-fold or greater rise in titre in only 10 out of the 21 pairs of sera. In 4 pairs no rise at all was found (Fig. 2).

The anti-viral (anti-V) complement-fixing antibody did not appear until the 5th–8th day of the disease but thereafter high levels were rapidly and constantly attained. Increases of 8- or 16-fold were common and even when the first serum was taken as late as the 8th day of the disease it was always possible to show at

least a 4-fold rise in titre. The highest titres were observed in samples of serum collected on or after the 13th day of the disease.

The level was maintained for about 7 weeks when a slow decline occurred: after 18 months this antibody was still detectable in amounts some four times greater than that present in the early days of the disease. Without exception, in every convalescent case of mumps the anti-viral antibody was raised significantly above the common initial titre of 4.

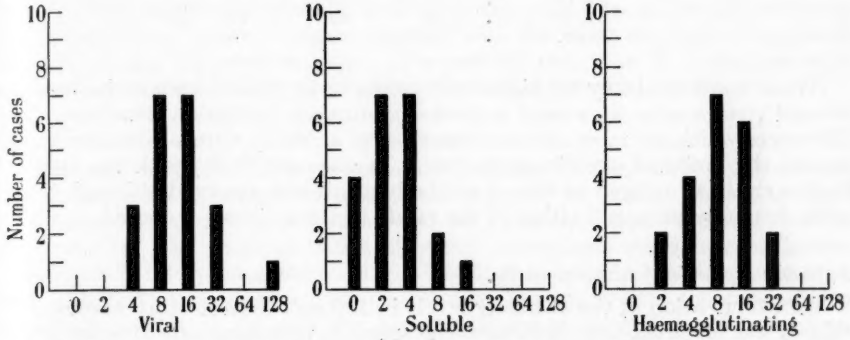


FIG. 2.—The -fold rise of antibody titre in 21 paired sera from cases of mumps estimated by both complement fixation methods and by haemagglutination inhibition.

Haemagglutination-inhibiting antibody levels with different virus strains.

With a view to determining whether differences of antigenic structure occur in strains of the mumps virus, and, if this is so, whether the variations are of practical importance in serological work, 18 acute sera (8 days or less) and 19 convalescent sera (9 days or more) were titrated by haemagglutination inhibition against three strains of the mumps virus. These sera included 15 paired samples. The differences between the end points when different virus strains are used are shown in Table I.

TABLE I.—*The Titres of the Same Sera in Haemagglutination Inhibition Tests with Three Different Strains of Mumps Virus.*

Sample.	Virus.	Titre. 16.	32.	64.	128.	256.	512.	1024.	2048.
Acute (Total = 18)	Enders	1	8	6	2	1	—	—	—
	James	—	4	10	4	—	—	—	—
	Paul	2	6	7	2	1	—	—	—
Convalescent (Total = 19)	Enders	—	—	—	3	7	4	4	1
	James	—	—	—	1	5	4	5	4
	Paul	—	—	1	1	3	5	9	—

When the increase in antibody titre of the paired sera was considered it was found that five of the patients showed quite different increments with the different viruses. Five other patients had identical antibody titres with each virus strain while in the remaining five an identical rise in titre was obtained with two strains.

TABLE II.—*The Rise of Haemagglutination Inhibition Antibodies against Three Different Virus Strains.*

Patient.	Fold-Rise. Enders Strain.	James Strain.	Paul Strain.
C. D.	8	4	16
M. Y.	8	16	64
B. L.	32	16	8
A. C.	32	64	16
N. S.	0	4	4

There was a tendency for higher end points to be obtained when the recently isolated virus strains were used in haemagglutination inhibition titrations. The differences which are more obvious when rise of antibody titre is considered were outside the limits of experimental error. In one case (N.S.) with the classical Enders strain as antigen no rise of antibody at all was observed although it was quite definitely present if either of the newly isolated viruses was used.

Antibody levels in healthy persons.

An examination of the antibody levels in 100 sera from healthy normal individuals was undertaken to determine the amounts persisting long after an attack of mumps. The figures obtained from haemagglutination inhibition and complement fixation tests on these sera from fourth year medical students, voluntary blood donors and new-born infants are shown in Table III.

TABLE III.—*Antibody Levels in Healthy Individuals.*

Reciprocal of serum dilution.	No. of sera examined with H.I. antigen.	No. of sera examined with Viral C.F. antigen.	No. of sera examined with Soluble C.F. antigen.
<4	—	25	38
4	—	13	12
8	—	18	3
16	1	11	1
32	9	1	—
64	20	1	—
128	45	—	—
256 or +	25	—	—
Total number of sera	100	69	54

In this sample of the population 64–70 per cent of the subjects possessed H.I. and anti-V complement-fixing antibodies in amounts considerably in excess of those in the susceptible subject. The absence of anti-S antibody from 70 per cent of the sera indicates that this substance disappears from the blood more rapidly than the other antibodies.

Of 54 fourth year medical students aged on an average 22 years, 32 gave a definite history of mumps in the past. All had been infected before the age of 16 and individuals known to have had the disease in the preceding 2 years were excluded. No exact estimate of the time interval between the onset of

clinical mumps and the time of serological testing was possible because only a few students could remember accurately their age when they were infected. In this group of 32 students 87 per cent of sera had H.I. antibody titres of 128 or over, 78 per cent had anti-V antibody titres of 4 or over, and only 12 per cent had anti-S titres of 4 or over.

Ten of the 54 students denied vigorously ever having had mumps; of these 6 (11 per cent) had no detectable antibodies and could be regarded as susceptible to mumps. The remaining four students had amounts of H.I. and anti-V antibodies which suggested definite infection in the past. In one anti-S antibody was present at a titre of 4 which, together with the other antibodies, suggested infection during the previous year. It is possible that these four students gave inaccurate histories or that they suffered from inapparent infections.

DISCUSSION.

The great majority of people suffer from mumps in their early years and are then immune for the result of their lives. Specific antibodies, augmented perhaps from time to time by re-exposure to the virus, persist in the circulation for long periods. The level at which they are maintained acquires considerable importance in interpreting the serological findings in cases of lymphocytic meningitis. Several years after recovery from mumps the antibody to the viral antigen may have a titre of 16 and the haemagglutination-inhibiting antibody may remain at 256. Table IV shows the titres of sera obtained at widely varying intervals after the onset of mumps.

The great value of the anti-S complement fixation reaction lies in the fact that a positive finding is frequently obtained during the first six days of the illness and that therefore timely diagnostic data are available for the clinician. Just as the presence of the Vi antibody in the serum so often indicates a current infection with the typhoid bacillus, so in the great majority of susceptible persons positive anti-S fixation with a serum dilution of 1 in 4 or more means an existing mumps infection.

The early appearance of the anti-S antibody and its rapid climb to a peak after fourteen days makes it uncommon ever to obtain from an active case of mumps a serum sample from which this antibody is entirely lacking. The difficulty of obtaining specimens of serum so spaced as to demonstrate a 4-fold rise in titre accounts for the absence of this diagnostic criterion in 10 out of 21 pairs of sera here examined. In most of the initial sera of these pairs the antibody level was already high.

The anti-viral complement fixation reaction has provided a constant and reliable check on the findings with the soluble antigen. Using this test titres of 8 or above were always obtained after the eighth day of the disease and whenever paired sera were examined it was always possible to demonstrate at least a 4-fold antibody rise (Fig. 2).

The presence of the anti-S antibody accompanied as it always is by the anti-V antibody indicates that infection probably occurred during the previous 9–18 months.

In Fig. 3 it is shown that sera collected during the first 7 days of mumps have higher anti-S than anti-V titres. From 8–14 days either antibody may reach a higher level, while from 15 days onwards the anti-V titres are almost invariably higher.

The simultaneous use of both of these complement fixation reactions with two or more suitably spaced serum samples gives consistent and reliable results from the beginning of disease onwards. Since the antigens remain stable for 6–9 months at 4° the methods are also of considerable practical utility.

TABLE IV.—*The Antibody Levels in 66 Sera from Cases of Mumps Estimated by Three Methods.*

Patient.	Sample No.	Days after onset.	H.I. antibody.		Complement-fixing antibody. Against viral antigen.		Complement-fixing antibody. Against soluble anitgen.		Neutralising antibody.	
			Titre.	Rise.	Titre.	Rise.	Titre.	Rise.	Titre.	Rise.
P. S.	1	1	32		<2		4		16	
	2	9	64		32	× 32	64	×16	256	
	3	32	256	× 8	16		16		512	× 32
	4	540	128		8		4		128	
R. S.	1	2	32		<4		4		16	
	2	15	256	× 8	32	× 16	16	× 4	256	× 16
	3	540	256		8		4		64	
I. Mc.	1	14	256		64		32		512	
	2	27	512	× 2	64		16		256	
	3	42	256		128	× 2	16		256	
	4	63	256		32		8	× 1	N.D.	
	5	353	256		32		8		64	
C. H.	1	3	32		<2		8		<16	
	2	10	512		8		32		128	
	3	17	1024	×32	16	× 16	32	× 4	512	× 64
	4	255	256		4		4		64	
J. S.	1	11	256		8		16		16	
	2	40	256		64		32	× 2	128	
	3	78	256	× 1	32	× 8	16		512	× 64
	4	270	128		16		8		64	
I. M.	1	8	256		8		32		256	
	2	19	512	× 2	32	× 4	32	× 1	1024	× 4
	3	26	512		8		16		512	
Y. M.	1	2	32		<2		16		32	
	2	16	256	× 8	32	× 16	16	× 1	256	× 8
S. W.	1	11	128		<4		8		256	
	2	16	1024	× 8	32	× 16	16	× 2	512	× 2
D. L.	1	5	64		4		4		N.D.	
	2	15	512	× 8	16	× 4	8	× 2	N.D.	
S. E.	1	3	16		4		16		16	
	2	14	256	×16	64	× 16	64	× 4	1024	× 64
J. D.	1	5	32		<4		4		32	
	2	20	128	× 4	256	×128	32	× 8	1024	× 32
J. W.	1	3	32		4		32		16	× 64
	2	12	512	×16	32	× 8	64	× 2	1024	
M. I.	1	5	<16		<4		4		32	
	2	13	256	×32	32	× 16	16	× 4	1024	× 32
I. B.	1	3	32		<4		<4		16	
	2	11	512	×16	32	× 16	<4	× 1	512	× 32
A. C.	1	5	64		<4		8		32	
	2	13	2048	×32	64	× 32	32	× 4	1024	× 32
B. L.	1	7	16		8		8		128	
	2	20	512	×32	256	× 32	32	× 4	1024	× 8
J. B.	1	2	32		<4		<4		<16	
	2	15	128	4	16	× 8	8	× 4	1024	×128
J. Do.	1	6	128		8		<4		32	
	2	19	1024	× 8	64	× 8	4	× 2	256	× 8
B. C.	1	6	128		<4		8		16	
	2	23	1024	× 8	32	× 16	16	× 2	256	× 16
I. M.*	1	4	128		8		8		32	
	2	12	1024	× 8	64	× 8	8	× 1	256	× 8
W. G.	1	5	64		8		8		32	
	2	11	1024	×16	64	× 8	16	× 2	256	× 8
J. J.*	1	8	64		16		8		128	
	2	15	256	× 4	512	× 32	16	× 2	1024	× 8
C. D.	1	5	32		<2		2		64	
	2	16	256	× 8	8	× 8	16	× 8	512	× 8
G. B.	1	5	128		8		16		64	
	2	16	512	× 4	64	× 8	32	× 2	1024	× 16
N. B.	1	13	512		16		32		256	
C. C.*	1	9	512		8		16		128	
H. L.	1	4	64		2		16		<16	
G. F.	1	8	64		4		16		32	
J. McN.	1	5	64		4		8		16	
S. Mc.	1	6	32		<2		4		32	
N. Ro.	1	3	32		4		4		N.D.	

* Meningo-encephalitis without parotitis.
N.D. = Test not done.

The antigenic stimulus which calls forth the production of anti-S antibody probably begins to take effect at the end of the second week of the long incubation period of mumps and thus precedes the action of the viral antigen—the virus elementary body—by some 6–8 days. There is here some indication of a position analogous to the soluble phase of the influenza virus postulated by Hoyle (1948). Certainly the S antigen behaves in quite a different manner and must be regarded as distinct from the virus elementary body which is so closely associated with haemagglutination and with the V complement-fixing antigen.

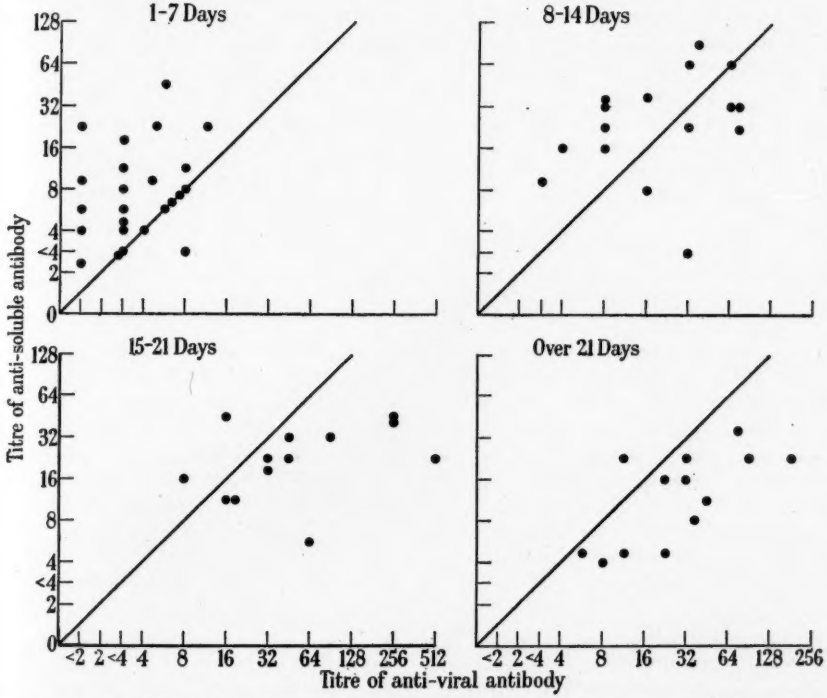


FIG. 3.—Correlation between antibody levels against S and V antigens at various intervals after the onset of mumps.

The haemagglutination inhibition test is almost as satisfactory as the complement fixation reaction and were it not for the instability of the antigen required it might conveniently be used in routine work. Although the sera were not treated with potassium periodate a significant rise in titre was demonstrated in 19 of 21 pairs of sera. The adoption of the technique of Robbins *et al.* (1949) in which 4 haemagglutinating doses of virus are added to each serum dilution has given uniformly satisfactory results. The larger amount of virus used and the different technique of virus titration employed by these authors is probably responsible for the undoubted advantage of their method over that of Lundbäck (1949).

During the first five days after the onset of mumps the antibody titre as estimated by the H.I. test is never above 64. The observation of a titre of 128 or above on a single serum from a patient who has never had mumps is highly suggestive of active infection and is often followed by a significant rise of antibody.

Although Leymaster and Ward (1948) demonstrated the value of the neutralisation test *in ovo* no attempt has been made since to apply the method to a serological study of this type. The test has proved to be reliable in the diagnosis of mumps. The appearance of the neutralising antibody on the sixth day of the disease and its increase and gradual decline have provided a useful yardstick with which to compare the fluctuations of the other antibodies. Although the test is technically formidable and is not feasible for routine work it may well be the most accurate guide to the susceptibility of the individual. Sera from normal persons susceptible to mumps do not prevent the infection of eggs when diluted to 1 in 32 or over; after the first week of the disease sera diluted to 1 in 128 or over are protective. Long after the infection has subsided the neutralising antibody is sustained at levels well above those found in acute stage sera.

In three cases the patients were known to have been in recent contact with mumps; no sign of parotitis were ever manifest and the clinical features were those of an acute meningo-encephalitis. Serological tests on sera from these patients gave unequivocally positive findings with considerable antibody rises in two cases where paired sera were obtained, and initial levels well above the normal in the third case.

It is not known what proportion of cases of aseptic meningitis of the lymphocytic type are caused by mumps virus. Alm (1951) used serological methods to show that in Sweden 8 per cent of 76 cases of this kind had a rising titre of antibody to this virus. An even higher incidence of 15 per cent was reported by Rasmussen (1946) in the U.S.A. in 374 soldiers who developed aseptic meningitis. A start has been made in the application of serological methods to the diagnosis of these cases in Edinburgh and so far eighteen patients have been investigated. The serological findings have so far been either negative or within the limits described for normal healthy people.

SUMMARY.

Three different methods have been used to estimate antibody levels in 66 samples of human serum taken during and after mumps.

Antibodies to the two complement-fixing antigens of mumps virus appear at constant intervals after the onset of infection. Those to the soluble (S) antigen appear early in the disease and reach a high level before the antibodies to the virus-bound (V) antigen begin to rise. This fact is of great value in establishing an early diagnosis in atypical cases of mumps by means of routine serological methods. Later the antibodies to the S antigen decrease at a rate considerably faster than that of other antibodies.

The neutralising antibody appears at the 5–6th day and is followed very closely by the haemagglutination-inhibiting antibody.

The haemagglutination inhibition (H.I.) test is reliable and consistent but the antigen remains stable for only one month at — 35°. In one third of the sera examined, significantly higher titres were obtained by this method when freshly isolated virus strains were used as antigens.

Our grateful thanks are due to Dr. Alexander Joe, Medical Superintendent of the City Fever Hospital in Edinburgh for access to the patients and their records, and for his great help in obtaining a major part of the material which forms the subject of this paper. We wish also to express our thanks to Professor T. J. Mackie for his advice and criticism. We are indebted to Mr. J. Sutherland for much technical help.

REFERENCES.

AIKAWA, J. K., AND MEIKLEJOHN, G.—(1949) *J. Immunol.*, **62**, 261.
ALM, L.—(1951) *Act. med. scand.*, supp. 263.
BANKS, H. S.—(1951) ' Modern Practice in Infectious Fevers.' London (Butterworth).
ENDERS, J. F., AND LEVENS, J. H.—(1948) ' Diagnostic Procedures for Virus and Rickettsial Diseases.' New York (American Public Health Association).
FELLER, A. E., AND JORDAN, W. S.—(1950) *J. Lab. clin. Med.*, **36**, 360.
HENLE, G., HARRIS S., AND HENLE, W.—(1948) *J. exp. Med* , **88**, 133,
HENDERSON, W,—(1952) *Lancet, i*, 386,
HOYLE, L,—(1948) *Brit, J, exp., Path*, **29**, 390,
KILHAM, L,—(1949) *Amer. J. Dis. Child.*, **78**, 324.
LEYMASTER, G. R., AND WARD, T. G.—(1948) *Amer. J. Hyg.*, **45**, 52.
LUNDBÄCK, H.—(1949) *Brit. J. exp. Path.*, **30**, 221.
RASMUSSEN, A. F.—(1946) *Rocky Mountain Conference on Poliomyelitis.* Denver, Colorado.
ROBBINS, F. C., KILHAM, L., LEVENS, J. H., AND ENDERS, J. F.—(1949) *J. Immunol.*, **61**, 235.

THE EFFECTS OF ETHYL ETHER ON SOME BIOLOGICAL PROPERTIES OF INFLUENZA VIRUS.

WILSON SMITH, H. COHEN, G. BELYAVIN AND J. C. N. WESTWOOD.*

From the Department of Bacteriology, University College Hospital Medical School, London, W.C.1.

Received for publication June 3, 1953.

THE biological properties of the influenza viruses have been studied intensively during recent years and some of them have been shown to be dependent upon specific reactive components of the virus surface. Practically nothing is known, however, about the virus architecture though it is probable that the spatial relationships of the various components concerned with biological activities like haemagglutination, complement fixation and enzyme action help to determine the efficiency of the virus unit as a whole. One obvious approach to an investigation of virus structure is the study of the effects of virus degradation by different chemical and physical agents. This paper reports the effects of ether treatment on the complement-fixing antigen, the haemagglutinin and the receptor-destroying enzyme of classical virus strains.

MATERIALS AND METHODS.

Virus preparations.

Influenza virus A (PR8) was used for most of the work but replicate experiments with Influenza B (Lee) established that results were applicable to both the major types of influenza virus. Both viruses were egg-adapted and were maintained by allantoic inoculation of 10-day embryonated eggs. Virus suspensions for experiment were obtained from infected allantoic fluid by adsorption on human group O red cells followed by elution into physiological saline.

Antisera.

Rabbits were inoculated subcutaneously with a single large dose of virus and bled at intervals thereafter. The sera were inactivated at 56° for 30 min. and stored in sealed ampoules at — 20°.

Haemagglutinin titrations.

These were done by the modification of the Hirst-Pickels photo-electric densitometer method described in a previous paper (Belyavin, Westwood, Please and Smith, 1951). In this method the densitometer readings are inversely proportional to the unagglutinated cell densities at the tube level traversed by the optical axis of the instrument. Except when specifically stated all red cell suspensions were prepared from the pooled blood of three fowls.

Complement fixation.

To measure the complement-fixing potency of an antigen preparation serial dilutions of both antigen and antiserum were mixed with a constant dose of complement. Both a "macro-" and a "micro-" technique were used. The former, employing a test volume of 0·5 ml. has previously been described (Smith, Westwood, Westwood and Belyavin, 1951).

* Seconded for virus research from the Staff of the Public Health Laboratory Service.

The micro-method (Belyavin, 1953) is identical in principle but permits the volume of each reagent to be reduced to 0·02 ml., the reagents being distributed by dropper pipettes on plastic sheets as described by Fulton and Dumbell (1949). Incubation is carried out in a humidity box and the fixation time is extended to 90 min. Thirty min. after the addition of standard volumes of 0·2 per cent sensitized sheep cells the plastic sheets are rocked on a centripetal rocker which brings any intact cells to the centres of the test drops as clearly visible dots or discs. The end-point, taken as complete haemolysis, is then easily detected by naked eye observation.

Antigen doses were adjusted from preliminary haemagglutinin titrations to commence at approximately 32 AD/ml. in the macro-technique and approximately 50 AD/ml. in the micro-technique.

When dilutions of both antigen and antiserum are used with a constant dose of complement to form a chessboard titration and the end-points for each row and column are plotted on a double logarithmic scale the area enclosed by the curves is proportional to the logarithm of the antigen concentration initiating the dilution series. This provides a reasonably accurate method for quantitative comparisons of the complement-fixing potencies of virus preparations before and after various types of treatment.

Ether treatment of virus.

"Aether puriss," B.D.H. was used. Five volumes of ether were added to one volume of virus suspension in a separating funnel. The mixture was shaken by a gentle to-and-fro motion for 2½ min. and the ether and aqueous phases then allowed to separate for a further 2½ min. This manipulation was repeated after which the ether and aqueous phases were either separated immediately or after being kept at 4° for various periods up to a maximum of 18 hr. The aqueous fraction was freed from residual ether by aeration. Any precipitate formed at this stage was removed by centrifugation and discarded as it was found to be completely inert in respect of both haemagglutination and complement fixation.

EXPERIMENTAL.

The Effect of Ether Treatment on Virus Haemagglutination.

Variability of effect.

The effect of ether treatment on the haemagglutinin titres of different PR8 virus preparations was extremely variable. In a number of experiments over 90 per cent of the initial haemagglutinating activity was destroyed but in others less than 30 per cent was lost. Many experiments were carried out to try to account for this variability; they included the use of purified "Analar" and peroxide-free ether, extractions at different pH values ranging from pH 6·0 to 7·9, extractions in the presence of citrate ions, and extractions with ether acidified with acetic acid. The effect of variations in the ambient temperature was also investigated. Consistent results were not obtained by any of these modifications. It was noted that repeated extractions of single virus preparations usually yielded approximately constant results although there was one exception when the same preparation gave haemagglutinin recoveries ranging from 70 to 5 per cent in three successive extractions. These were done, however, on different days and we feel that the variable factor lies in the virus itself rather than in the technique of ether extraction. This possibility remains to be explored.

The qualitative change in the haemagglutination titration curve.

The haemagglutination curve obtained by plotting densitometer readings against an adequate range of virus dilutions always shows a marked optimum of virus concentration; *i.e.*, all tubes containing either higher or lower con-

centrations than the optimal tube show less sedimentation of the fowl red cells at the standard reading time and hence give lower densitometer readings. Thus excess virus clearly causes a well-marked zone of haemagglutination inhibition analogous to the excess antigen inhibition zone of serum precipitation reactions. Provided that enough activity remains to allow demonstration of an inhibition zone any reduction of the haemagglutinin content of a virus preparation would be expected to shift the curve bodily to the left without any change in the densitometer reading at the optimal point. This is indeed the case when haemagglutinin activity is reduced by heat treatment. It is also the case with ether treatment provided that the treatment results in 90–95 per cent reduction. When however the reduction is relatively slight the curve assumes a different form. It is shifted downwards instead of to the left, with a very much lower densitometer reading at the optimal point. The results of a typical experiment with untreated, heated and ether-treated virus are illustrated in Fig. 1.

The variability of different fowl cells.

For quantitative determinations of this qualitative effect of ether treatment on virus haemagglutinin activity maximum densitometer readings could not be used as such, because densitometer readings are not linearly related to cell concentrations. When cell concentrations are low quite small changes of suspension density produce large changes in densitometer readings with the result that small effects tend to be spuriously magnified. To avoid this a simple calibration curve was constructed for the conversion of densitometer readings to percentage cell concentrations. Figures thus obtained actually represent cell concentrations at the optical level of the densitometer and are not strictly accurate assessments of the relative proportions of agglutinated and unagglutinated cells. They are however sufficiently accurate for the purpose. Throughout this paper the term " optimal reading " is used to designate the highest densitometer reading of a titration curve while the term " optimal concentration " refers to the concentration of unsedimented cells in the tube giving the optimal reading. The two optima are of course inversely related.

Four different virus batches were extracted with ether. Ether-treated and untreated samples of each batch were titrated in parallel against red cell suspensions obtained from 8 fowls. The optimal concentrations are tabulated in Table I.

TABLE I.—*Effect of Ether Treatment on Virus Haemagglutination.*

Fowl cells No.	Passages of PR8 virus. ES 154. Unt.	ES 154. Eth.	ES 165. Unt.	ES 165. Eth.	ES 169. Unt.	ES 169. Eth.	ES 171. Unt.	ES 171. Eth.
1531	0·11	0·155	—	—	—	—	—	—
1532	0·11	0·155	—	—	—	—	—	—
1533	0·08	0·155	0·085	0·19	0·08	0·15	0·085	0·135
1534	0·12	0·225	—	—	—	—	—	—
1536	0·11	0·20	0·09	0·225	0·08	0·225	0·075	0·18
1537	0·095	0·175	—	—	—	—	—	—
1538	0·09	0·145	—	—	—	—	—	—
1500	—	—	0·085	0·225	0·07	0·240	0·085	0·145

Figures give the " Optimal concentrations," for explanation of which term see text.

The downward shift of the titration curves produced by ether treatment is indicated by the appreciably higher optimal concentrations for the ether-treated samples of each virus batch as compared with those for the corresponding untreated samples. This happened with the cells of every fowl tested. The different cell suspensions however showed interesting differences of behaviour. It is well known that the cells of different fowls may vary in the readiness with

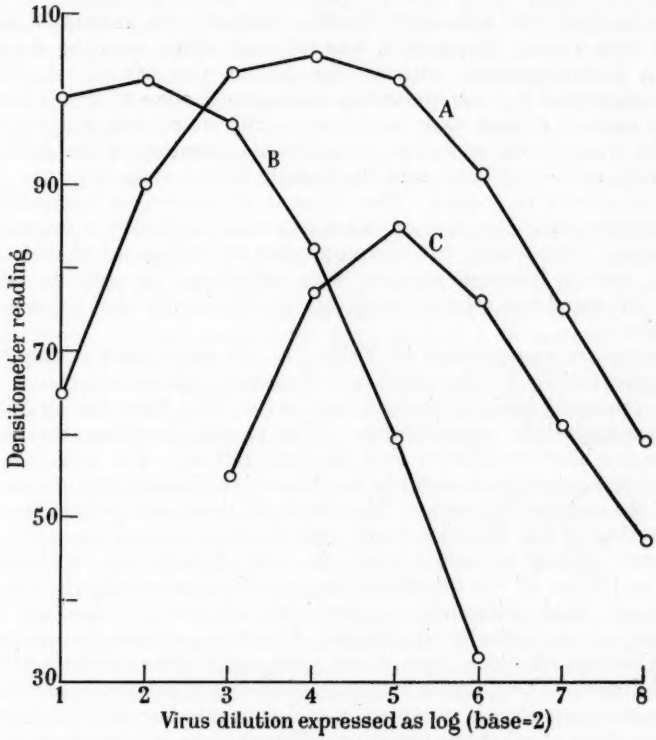


FIG. 1.—Haemagglutinin titration curves.
A. Untreated virus.
B. Heated virus.
C. Ether-treated virus.

which they are agglutinated by influenza viruses but those used for the experiment behaved with surprising uniformity against the four untreated viruses. In contrast the optimal concentrations against each ether-treated virus varied considerably and the relatively high or low susceptibility of each individual fowl's erythrocytes was maintained consistently in tests of the four different virus preparations. The relative susceptibilities of two cell suspensions against untreated and ether-treated virus may actually vary in opposite directions so that the suspension giving the lower optimal concentration with untreated virus

proves to be much less readily agglutinable than the other by the same virus after its ether extraction.

Three breeds of fowls were represented among the blood donors, White Leghorn, Brown Leghorn and Light Sussex, but so far as could be ascertained breed was not a factor in this variability of red cell susceptibility.

Consistent behaviour of cells of individual fowls.

In order to study the behaviour of cells obtained from individual fowls over a period of time a virus preparation was required which could be depended on to maintain haemagglutinin activity throughout the period. A PR8 virus eluate was distributed in 1 ml. quantities in ampoules, some of which were freeze-dried, some kept at 4° and some stored at — 20° after being rapidly frozen at — 76°. The freeze-dried virus lost considerable haemagglutinin activity in 3 days and much more in 7 days, and the rapidly frozen virus kept at — 20° lost over half its activity in 3 days. The virus at 4° showed no appreciable diminution of potency in 7 days and on ether treatment still gave a typical lowered titration curve. This batch was therefore used for the series of tests. At approximately weekly intervals samples were withdrawn in order to titrate the virus, both untreated and ether-treated, against the freshly bled red cells of three individual fowls.

The results are summarized in Table II. At each weekly test the three optimal concentrations for the untreated virus were almost identical. By itself this would apparently indicate that the cells of the three fowls were indistinguishable in haemagglutinin susceptibility. The parallel readings for the ether-treated samples however showed that this was not so. Not only did the cells differ widely in sensitivity at each test but the order of sensitivity was maintained throughout the series of five tests. Thus while all three cell suspensions regularly showed lowering of the titration curve against ether-treated virus as compared with the curve against untreated virus, the cells of two fowls consistently gave very high, and those of the third fowl consistently gave relatively low, optimal concentrations. Such differences as are shown by various bleedings from the same fowl are not statistically significant. It is thus evident that red cell sensitivity to ether-treated virus depends on some stable characteristic of the individual fowl donor. The uniform response of the virus eluate to ether treatment on five separate occasions is noteworthy and supports our contention that variability of the effect of ether treatment on different virus batches depends on some unknown variable in the virus itself.

TABLE II.—*Variable Susceptibility of Cells of Different Fowls to Ether-treated Virus.*

Virus.	Fowl cells.	Date of test. 17.vii.50.	21.vii.50.	27.vii.50.	11.viii.50.	18.viii.50.
Untreated	1500	0·09	0·085	0·09	0·08	0·065
	1533	0·085	0·09	0·085	0·085	0·07
	1536	—	0·09	0·085	0·08	0·075
Ether-treated	1500	0·205	0·255	0·255	0·24	0·205
	1533	0·115	0·190	0·195	0·175	0·180
	1536	0·25	0·225	0·260	0·24	0·255

Figures = optimal concentrations.

The significance of lowering of the virus titration curve.

It is generally assumed that any reduction of haemagglutination titre resulting from some treatment of the virus signifies that destruction of some of the haemagglutinin has occurred. This is not necessarily so. Any alteration of position of the titration curve in relation to either of its co-ordinates must affect the titre. If titre reduction is due to a reduction of the amount of haemagglutinin in the virus preparation, either by simple dilution or by partial haemagglutinin destruction, the shift of the curve will be to the left. This implies that titre reduction associated with downward displacement of the titration curve must depend upon some kind of haemagglutinin change distinct from quantitative reduction. It appears therefore that one effect of ether treatment may be a qualitative change in the virus receptors. Actual haemagglutinin destruction may also occur to a variable extent; so that a reduction of titre following ether treatment may represent either a quantitative or qualitative change or a summation of both.

The validity of comparisons based on haemagglutination titrations rests on rigorous standardization of the method used so that experimental errors can be kept within small and defined limits. It is essential that the time allowed for cell sedimentation in each tube before taking the densitometer reading should be kept constant. In our method the time chosen is 75 minutes. Within any arbitrarily selected time any factor which slows the haemagglutination reaction will produce downward displacement of the titration curve. It seemed likely that the qualitative change in a virus produced by ether treatment might be such as to cause increase of haemagglutination reaction time. The temporal evolution of titration curves was therefore studied.

A series of replicate titrations was carried out on virus untreated and ether-treated. Dilutions were prepared in bulk and distributed to give several identical replicate dilution series. Each replicate was read after the lapse of a different time interval for cell sedimentation, ranging from 15 to 105 min. The series of titration curves thus obtained is illustrated in Fig. 2.

At 15 min. curves for untreated and ether-treated virus are almost identical and are flat because no appreciable cell sedimentation has yet occurred. At 45 min. they are parallel but widely separated. Subsequently both rise steadily so that both the untreated and ether-treated viruses give a series of ascending roughly parallel curves but at any give time the ether-treated virus curve is considerably lower than the corresponding untreated virus curve. It is clear therefore that the effect of ether treatment of virus on its haemagglutinating activity is operative in the early stages of the reaction and that in later stages the reaction proceeds normally. In the experiment illustrated the retardation time was approximately 30 min. so that ether curves for 75 and 105 min. are superimposed over the "untreated" curves for 45 and 75 min. respectively. In a second experiment using the cells of a different fowl the retardation time was about 15 min. and similar experiments with virus B (Lee) showed that its haemagglutinating activity was affected by ether treatment in the same way though usually with a shorter retardation time.

Given sufficient time all the cells of a suspension settle out leaving a clear supernatant fluid. The acceleration of sedimentation caused by virus is due to the formation of cell aggregates of density greater than that of discrete cells so that the major factors which determine the time relations of the reaction are

speed of aggregate formation and mean aggregate size. It seemed probable that both factors would be affected by the presence of virus-insensitive cells in a cell suspension and that the ether retardation phenomenon might be due to variability of sensitivity to ether-treated virus among fowl red cells. Experiments showed however that this is not the true explanation. The rates of development of titration curves were studied using an untreated virus against several

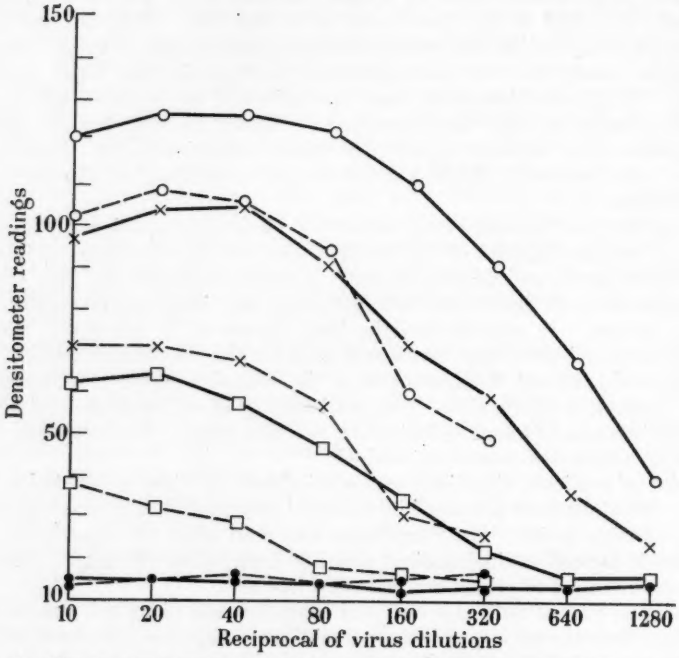


FIG. 2.—The temporal evolution of haemagglutinin titration curves with untreated and ether-treated virus.

●——● 15 minute readings.
⊡——⊡ 45 „ „
×——× 75 „ „
⊙——⊙ 105 „ „

Unbroken lines = untreated virus.
Broken „ = ether-treated virus.

cell suspensions containing various proportions of sensitive and unsensitive cells, the latter being obtained by means of purified *V. cholerae* receptor-destroying-enzyme prepared by the method of Burnet and Stone (1947). It was found that in such systems acceleration of sedimentation begins just as quickly as with normal cell suspensions due to the aggregation of the virus-sensitive cells which settle out to leave a slowly falling column of insensitive cells in suspension. This results in a deceleration of the reaction at a fairly early stage, a phenomenon never observed in ether-treated virus haemagglutination. In an ether-treated virus system acceleration of sedimentation is delayed but once having begun the

reaction proceeds throughout at the normal rate, indicating that the cell aggregates form more slowly than in an untreated virus system but that when formed they are of normal size. Possible reasons for this slower reactivity are discussed below.

The Effect of Ether Treatment on the Virus Complement Fixation Antigen.

While this work was in progress Hoyle (1950) reported that the complement-fixing potency of influenza virus was reduced by ether treatment. Our findings confirm this but their chief interest is that they show a dissociation of the effects of ether treatment on the virus complement-fixing potency and the haemagglutination activity in a manner directly contrary to that induced by heat inactivation. It should be emphasized that the complement-fixing antigens concerned are the strain-specific surface antigens of the virus elementary bodies, not the soluble antigens which are common to all strains within each of the major types A and B. The virus preparations used were purified eluates prepared from infected allantoic fluids; such preparations are free from demonstrable soluble antigen.

Saline eluates of PR8 virus were extracted with ether by the method described above. The ether phase was evaporated at 30–35° under reduced pressure until the small fluid residue was devoid of any smell of ether; this residue was then made up to the original volume with physiological saline. Some precipitate always formed in the aqueous phase; this was removed by centrifugation and re-suspended in the original volume of saline. The clear supernatant fluid from the aqueous phase was considered to contain the degraded virus particles. The three fractions and a sample of the untreated virus eluate were tested for complement-fixing potency and were also titrated in parallel for haemagglutinin. The results of four experiments are summarized in Table III.

TABLE III.—*Effects of Ether treatment on C.F. Potency and Haemagglutinin Activity.*

		Complement fixing potency.*				Haemagglutinin titres.				
			After ether extr.					After ether extr.		
Virus preparation.	Time of ether contact.	Before ether extr.	Aqueous layer.	Ether layer.	Ppt.	Fowl cells used.	Before ether extr.	Aqueous layer.	Ether layer.	Ppt.
PR8/ES133	10 min.	30	20	0	0	pool	680	>256	6·6	<4
PR8/ES133	Overnight	22	6	0	0	pool	765	469	17	6
PR8/ES153	,,	26	13	nt	nt	1532	482	120	nt	nt
						1536	526	63	nt	nt
PR8/ES154	,,	28	4	nt	nt	1532	523	109	nt	nt
						1536	485	<40	nt	nt

* The units of potency are arbitrary and are expressed on a logarithmic scale.

0 = no activity.
nt = no test.

In view of the effects of ether treatment on the haemagglutination reaction it is obvious that direct quantitative comparisons between residual haemagglutinin and residual complement-fixing antigen are not valid. Nevertheless, even when haemagglutinin titrations were done with fowl cells least sensitive to ether-treated virus the reduction of complement-fixing potency was never less than the

reduction of haemagglutinin activity and was usually very much greater. Sometimes indeed preparations were obtained which were entirely devoid of complement-fixing activity while retaining appreciable haemagglutinin activity. The reverse effect never occurred. Yet this is precisely the effect which was always obtained by sufficient heat treatment of the virus. PR8 eluates were heated at 60° until all detectable haemagglutinin had been destroyed (titre < 2); they were then found to have retained about a quarter of their initial complement-fixing potencies. Eluates were heated at 56° for short periods so as to leave considerable residual haemagglutinin activity. Unheated and heated samples were then diluted so as to contain the same haemagglutinin concentration, namely 32 AD/ml., and compared directly in complement fixation tests. Two such experiments are summarized in Table IV. In both, the heat degradation caused an apparent increase of complement-fixing potency. Such apparent increase would of course result from a more rapid destruction of haemagglutinin than of complement-fixing antigen and this explanation was further supported by experiments in which virus was heated for various periods at different temperatures and complement fixation tests done in parallel on all the preparations after adjusting them to equivalent haemagglutinin content. The ratio of complement fixation units to AD showed a steady increase with increasing heat treatment.

TABLE IV.—*Relative Resistance of C.F. Antigen to Heat Inactivation.*

	Haemagglutination titres.		Complement fixing potency.*	
Virus preparation.	Before heating.	After heating.	Before heating.	After heating.
PR8	341	248	11	15
	408	147	10	12

* After adjustment of antigen to 32 AD/ml. using heated HA titre.

The Effect of Ether Treatment on the Receptor-Destroying Enzyme of the Virus.

Previous work had shown that ether-treated virus was more sensitive to normal serum inhibitor than untreated virus, although it retained considerable enzymic activity. This was advanced as conclusive evidence that the "indicator state" could not be dependent on destruction of virus enzyme, as was then generally believed (Smith *et al.*, 1951). Table V summarizes experiments in

TABLE V.—*Conversion of Virus to Indicator State by Ether-treatment.*

Exp. No.	Virus strain.	Inhibitory titres of normal rabbit sera against viruses		
		Untreated.	Heated.	Ether-treated.
1	PR8	<40	500	116
2	,,	<40	344	265
3	,,	32	377	1006
4	,,	<40	—	320
5	,,	<20	—	208
6	,,	<40	160	320
7	PR8/X	<40	<40	160
8	,,	<40	<40	320

PR8/X = a mutant of PR8 (Smith *et al.*, 1951). All the ether-treated preparations were shown to be enzymically active.

which ether-treated, heat-treated and untreated viruses were tested against normal rabbit serum. Without exception the ether treatment converted virus to the " indicator state " and in several instances produced a much more effective indicator virus than did heat treatment.

The function of the virus enzyme in the processes of infection has been the subject of much speculation but is still unknown. That it must play an important rôle is suggested by the fact that its destruction is invariably associated with loss of infectivity. Further study of its dissociation from haemagglutinin activity as a result of ether treatment was clearly indicated.

Quantitative assessments of the enzymic activity of virus preparations were made by measuring the proportion of virus which eluted after its adsorption on red cells. Packed washed fowl cells were added to equal volumes of virus, untreated and ether-treated, to give final cell concentrations of approximately 4 per cent. Adsorption was allowed to proceed for 30 min. at 4° ; then the cells were centrifuged out and re-suspended in saline to the original volume. The suspensions were incubated at 37° for 2 hr. after which the cells were removed by centrifugation. The supernatants were titrated in parallel for haemagglutinating activity.

The results of several such experiments on both PR8 and Lee viruses are presented in Table VI. They show that neither short (10 min.) nor prolonged (overnight) ether treatment markedly impaired the eluting powers of PR8 virus. This was in marked contrast to the behaviour of Lee virus, for which even 10 min. contact with ether caused at least 70–75 per cent reduction of eluting power and usually complete loss.

TABLE VI.—*Retention of Enzyme by Ether-treated Virus.*

Virus strain.	Passage No.	Period of ether contact.	Haemagglutination titres (ether-treated). Before absorption.	Haemagglutination titres (ether-treated). After elution.	Percentage yield on elution.
Type A	ES 157	10 min.	930	832	89
	ES 157	Overnight	452	395	88
	ES 161	10 min.	417	389	93
	ES 165	Overnight	125	94	75
	ES 169	10 min.	133	144	100
	ES 171	„	217	77	36
PR8 mutant	EX 182	10 min.	996	862	86
Type B " Lee "	EB 9	10 min.	105	0	0
	EB 13	„	160	30	18
	EB 14	„	124	<20	—
	EB 15	„	391	109	28

This method of estimating enzymic activity has a serious limitation. It depends on the retention of haemagglutinin activity by the eluted virus particles. Any virus which had suffered loss of its haemagglutinin receptors during the process of elution would be undetectable. Hence a dissociation of haemagglutinin and enzyme susceptibility to ether would tend to be obscured.

As an alternative the direct method of Lanni, Lanni and Beard (1951) for measuring enzymic activity was used. Virus preparations were mixed with egg

white and held at room temperature to allow progressive destruction of the egg white inhibitor. Aliquots were removed at various intervals and boiled for two min. to inactivate the virus. Finally all samples were titrated in parallel for residual inhibitor using heated PR8 virus as the indicator virus. It was found that within a certain range of virus dosage there was a linear relationship between inhibitor destruction and time so that a direct measurement of reaction velocity was possible. By this means it could be shown that ether-treated PR8 virus retained far more enzymic activity than could be accounted for by the residual haemagglutinin content. Clearly the virus haemagglutinin had been reduced preferentially in relation to the virus enzyme. Incidentally it was shown that the eluted ether-treated virus was completely non-infective ; an unknown infectivity factor must thus be more susceptible to destruction by ether than are haemagglutinin, complement-fixing antigen and virus receptor-destroying enzyme.

DISCUSSION.

Three important biological activities of the influenza viruses, specific complement fixation, haemagglutination and enzymic destruction of host cell receptors, are dependent on the integrity of surface components, but it is still unknown how far these components are independent or linked together in composite structures. It has been suggested that the enzyme may be an essential part of the virus haemagglutinin receptors which thus serve the dual purpose of cell-virus linkage and subsequent cell receptor destruction to facilitate cell penetration. Certainly separation of enzyme from the virus particle has not yet been accomplished. Our results, however, prove that haemagglutinin inactivation is not necessarily associated with loss of enzyme. Whereas virus treatment by heat or ultra-violet light invariably produces the inactivation sequence enzyme-haemagglutinin-C.F. antigen, exactly the reverse order of inactivation results from ether treatment. This suggests that the three types of biological activity may be functions of specific and distinct structures on the virus surface. Our results could equally well be due to the preferential destruction or distortion of subsidiary parts of a single complex surface entity by the different inactivating agents employed. If increased susceptibility of heated virus to non-specific inhibitors is a function of surface distortion as claimed by Smith and Westwood (1950) it follows that ether treatment must also induce such distortion because one of its effects is conversion of the virus to the indicator state. Sterically different surface distortions induced by different inactivating agents would adequately account for the observed phenomena.

A satisfactory interpretation of the qualitative change of haemagglutination behaviour induced by ether treatment demands a fuller elucidation of the mechanism of the optimal-reading phenomenon. This phenomenon is not merely an example of the immunological optimal proportions reaction dependent upon equivalence of antigen and antibody, for haemagglutination always appears first in the tube containing the highest concentration of virus. The rate of sedimentation in this tube, however, is outstripped by that occurring in the optimal tube. Neither can the inhibition zone be due to rapid enzyme action on red cell receptors when virus is present in excess, because such zones also occur in titrations carried out at 4°. As yet we have no satisfactory explanation to offer.

The evidence suggests that one effect of ether treatment of virus is to slow the speed of virus-red cell union. The most obvious way in which this could be

achieved in a mixture of fixed proportions of virus and cells would be by total inactivation of a proportion of the virus particles. That this is not the true explanation of the ether effect is clear from the absence of any shift to the left of the full haemagglutinin titration curve in many of our experiments. The speed of union and resulting aggregate formation however might equally be affected by the partial destruction of the receptors on each individual virus particle. If so, every particle would still be capable of linkage to red cells but enough linkages for aggregate formation would take considerably longer than with normal virus. It is possible that this intermediate stage always occurs in the inactivation of individual virus particles but that with heat treatment invariably, and with ether treatment sometimes, the phase of partial degradation is overshadowed by the rapidity with which it passes on to the phase of complete inactivation. An alternative explanation is the hypothesis of steric hindrance from surface distortion (Smith and Westwood, 1950). Extraction of ether-soluble lipids from the virus cannot fail to produce surface distortion and this may be of a type sterically different from that induced by heat. Quite small differences may well determine whether the receptors are unable to unite with the red cell receptor or are still able to do so though with difficulty. The highly variable effects of ether treatment would not be difficult to understand on this basis for the precise pattern of the induced distortion would depend on the original surface pattern of the treated virus. Our scanty knowledge of the phenomena associated with virus replication makes it extremely improbable that the surface pattern remains completely static throughout the reproduction cycle so that no matter how rigorously cultural conditions are standardized different batches of one and the same virus strain are unlikely to be absolutely identical. The complement fixation reaction, on the other hand, is not dependent on the pattern of the antigen in relation to other surface components and so is uniformly affected by ether treatment.

The anomalous haemagglutination behaviour of ether-treated virus is of some practical significance. With any method of haemagglutinin titration in which readings are taken before completion of cell sedimentation, the titre must be affected by the velocity of the reaction. The fact that reaction velocity may be affected by qualitative changes in a virus, independent of any quantitative reduction of its haemagglutinin, introduces a source of possible error into estimations by the photo-electric densitometer method. The precision of this method may therefore sometimes have to be sacrificed in favour of cruder methods in which titres are unaffected by reaction velocity.

SUMMARY.

The effects of ether treatment of influenza virus were variable in spite of rigorous standardization of experimental conditions. This is probably due to slight variation in different batches of a single virus strain.

The inactivation sequence with ether treatment is, complement fixation-haemagglutination-enzyme activity, which is the reverse of the sequence induced by heat or ultra-violet light inactivation.

Ether treatment causes a qualitative change of virus haemagglutination behaviour irrespective of any quantitative reduction of haemagglutinin. Speed of cell aggregate formation and hence of sedimentation is reduced. This affects

the position of the titration curve in relation to its co-ordinates in a manner different from that produced by quantitative reduction and hence also affects the apparent titre of the virus.

Some of the implications of the effects of ether treatment of virus are discussed.

One of us (H. C.) worked with a Medical Research Council Research Studentship. Expenses of the work were defrayed from a Medical Research Council grant.

REFERENCES.

BELYAVIN, G., WESTWOOD, J. C. N., PLEASE, N. W., AND SMITH, W.—(1951) *J. gen. Microbiol.*, **5**, 546.
Idem—(1953) *J. Hyg., Camb.* (in press).
BURNET, F. M., AND STONE, J. D.—(1947) *Aust. J. exp. Med.*, **25**, 227.
FULTON, F., AND DUMBELL, K. R.—(1949) *J. gen. Microbiol.*, **3**, 97.
HOYLE, L.—(1950) *J. Hyg., Camb.*, **48**, 277.
LANNI, F., LANNI, Y. T., AND BEARD, J. W.—(1951) *J. Immunol.*, **66**, 169.
SMITH, W., AND WESTWOOD, J. C. N.—(1950) *Brit. J. exp. Path.*, **31**, 725.
Idem, WESTWOOD, M. A., WESTWOOD, J. C. N., AND BELYAVIN, G.—(1951) *Ibid.*, **32**, 422.

A SEROLOGICAL PROCEDURE THEORETICALLY CAPABLE OF DETECTING INCOMPLETE OR NON-PRECIPITATING ANTIBODIES TO SOLUBLE PROTEIN ANTIGENS.

R. R. A. COOMBS, A. N. HOWARD AND L. S. MYNORS.

From the Departments of Pathology and Medicine, University of Cambridge.

Received for publication May 21, 1953.

In an earlier paper (Coombs, Howard and Wild, 1952) a procedure was described for measuring antibodies to soluble protein antigens by an agglutination reaction. The method entailed the previous conjugation of the protein antigen with the human incomplete Rh antibody by means of tetrazotised benzidine. The anti-protein serum was then tested for its ability to agglutinate red cells sensitized with the conjugated incomplete antibody.

This work was a preliminary stage to the development of an *in vitro* serological test capable of detecting incomplete or non-precipitating antibodies to soluble protein antigens. It should be possible to detect such antibodies in human serum if a rabbit incomplete anti-red cell antibody were available with which the protein antigen could be conjugated. Linked to such an antibody the protein could be fixed to the surface of red cells which would nevertheless remain stable in suspension. This cell system could then be used to absorb human incomplete antibody to the conjugated protein. The absorption could be detected finally by an agglutination reaction using rabbit anti-human globulin serum. This procedure is illustrated diagrammatically in Fig. 1.

There are three distinct stages in this procedure ; first, the preparation of the *protein antigen-red cell unit* ; second, exposure of the antigen cell unit to the human serum under investigation and lastly the addition of rabbit anti-human globulin serum as a *detector system* for the presence of absorbed human incomplete antibody.

At present we have no suitable rabbit anti-red cell incomplete antibody, but a stable antigen-red cell unit may be achieved using an ordinary potentially agglutinating rabbit anti-red cell antibody if advantage is taken of a property of the red cells of certain oxen. These cells have the property of remaining unagglutinated despite being sensitized with antibody. The serological behaviour of these so called " inagglutinable " bovine red cells has been described by Coombs, Gleeson-White and Hall (1951), who showed that the sensitized cells could be made to agglutinate by building out a chain of anti-globulin and globulin molecules from the globulin of the antibody sensitizing the cells.

For purpose of illustration these cells may be thought of as having hypothetical " prongs " protruding from the cell surface which prevent the cells coming sufficiently close together to allow the antibodies adsorbed primarily on their surfaces to link across from cell to cell.

Because of this property such cells may be used to obtain a stable protein antigen-red cell unit (Fig. 2). With this unit it is possible to detect complete or incomplete antibodies to the particular protein antigen. The scheme shown in Fig. 2 outlines the stages involved in the demonstration of anti-protein antibodies in human serum. It may be seen that the procedure is somewhat longer than that represented in Fig. 1. Demonstration of the adsorption of human antibody is not possible by a single application of rabbit anti-human globulin serum.

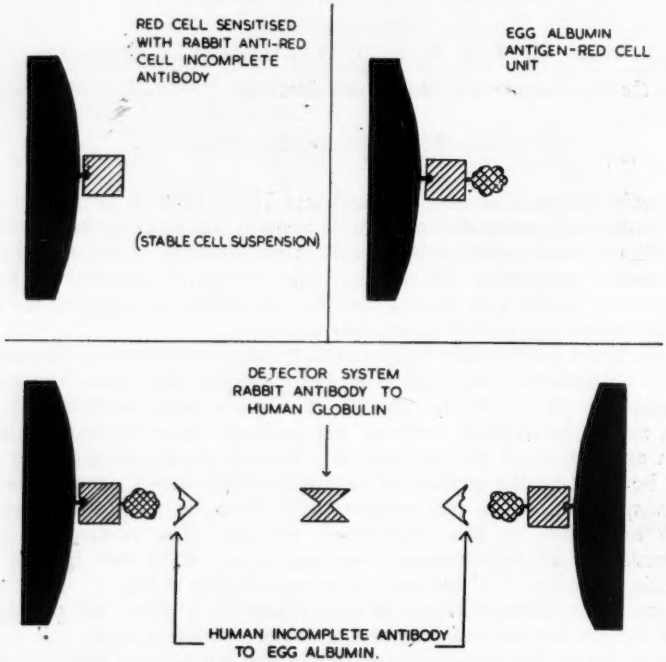

RED CELL SENSITISED WITH RABBIT ANTI-RED CELL INCOMPLETE ANTIBODY
(STABLE CELL SUSPENSION)
EGG ALBUMIN ANTIGEN-RED CELL UNIT
DETECTOR SYSTEM RABBIT ANTIBODY TO HUMAN GLOBULIN
HUMAN INCOMPLETE ANTIBODY TO EGG ALBUMIN.

FIG. 1.—Method envisaged for the serological detection of human incomplete antibodies to a soluble protein antigen such as egg albumin (for key see p. 531).

It is necessary with this system to build up a longer anti-globulin-globulin chain before the further addition of anti-globulin serum is able to effect agglutination.

By this method it is possible to measure, using a serial titration, anti-protein antibodies in the sera of different animal species. The demonstration is not dependent on any secondary agglutinating property of the antibody nor on any complement-fixing property but simply on the fact that the antibody is a globulin and combines with the antigen. For this reason the method should be able to demonstrate so-called incomplete or non-precipitating as well as ordinary complete antibodies. The method despite its many stages is specific and sensitive and has been used, as described in this paper, to demonstrate antibodies to egg albumin in the sera of the guinea-pig, dog and man, and antibodies to the protein of horse dandruff in the sera of guinea-pigs and man.

MATERIALS AND METHODS.

Materials for the preparation of the Protein Antigen-Red Cell Unit.

Egg albumin.—This was prepared as in our previous paper (Coombs *et al.*, 1952).

Horse dandruff protein.—This was prepared according to the method described by Squire (1950). It was kept freeze-dried till used.

Rabbit anti-bovine red cell serum.—This was a pool from five rabbits bled 10 days after a single course of inoculations of washed bovine cells. The course consisted of five intra-

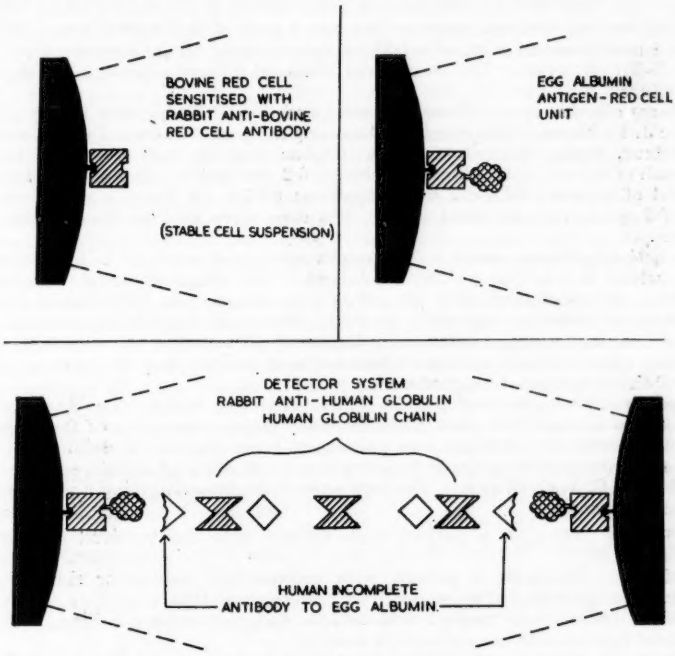

BOVINE RED CELL SENSITISED WITH RABBIT ANTI-BOVINE RED CELL ANTIBODY
(STABLE CELL SUSPENSION)
EGG ALBUMIN ANTIGEN-RED CELL UNIT
DETECTOR SYSTEM
RABBIT ANTI-HUMAN GLOBULIN
HUMAN GLOBULIN CHAIN
HUMAN INCOMPLETE ANTIBODY TO EGG ALBUMIN

FIG. 2.—Method described in this paper for the serological detection of human incomplete antibodies to a soluble protein antigen such as egg albumin (for key see p. 531).

peritoneal injections of 1 ml. of a 10 per cent washed cell suspension. It is important to use serum after a single course of injections as the serum after subsequent courses was found to agglutinate to a moderate degree even the "inagglutinable" class of bovine red cells.

Bovine red cells.—Bovine red cells of any class may be used for the inoculation of the rabbits, but for the *in vitro* testing only cells of the "inagglutinable" class may be used (see Coombs *et al.*, 1951).

Rrocedure for Conjugating both Egg Albumin and Horse Dandruff Protein with the Protein Molecules of the Rabbit Anti-bovine Red Cell Serum.

The conjugation was achieved in the same way as in our previous paper (Coombs *et al.*, 1952) using tetrazotized benzidine. A solution of the benzidine tetrazonium chloride was added, on the calculated basis of 8 mol. per 2 mol. protein, to mixtures of 5 ml. serum with 1 ml. 10 per cent egg albumin solution and 5 ml. serum with 2 ml. 10 per cent solution of horse dandruff protein. The solutions were then treated as described in the previous paper.

Materials used in the Detector Systems.

Rabbit anti-globulin sera.—Rabbit anti-human globulin, anti-guinea pig globulin and anti-dog globulin sera were prepared by inoculating rabbits with either whole or alum-precipitated serum. The anti-sera after heat inactivation were absorbed free of antibodies for bovine red cells.

Normal serum globulin of man, the guinea-pig and the dog.—Normal sera were used as the source of globulin from the different animal species. Each serum was heat-inactivated and absorbed free of antibodies to bovine red cells.

Sera Examined for Antibodies to Egg Albumin or Horse Dandruff Protein.

Guinea-pig anti-egg albumin serum.—This was a pool of 2nd course sera. Each course consisted of 5 injections of 1·5 ml. of a 1·25 per cent solution of egg albumin given intraperitoneally at 3-day intervals. The serum was obtained 9 days after the last injection by cardiac puncture.

Dog anti-egg albumin sera.—Three sera were examined. These were kindly given to us by Professor J. R. Marrack who received them originally in 1950 from Dr. P. Grabar of the Institut Pasteur, Paris. We hear from Dr. Grabar that the dogs received 4 intravenous injections each of 0·5 ml. of an alum-precipitated 0·2 per cent solution of egg albumin given over a period of a week, followed by 4 injections of 1·5 ml. for the second week and 4 injections of 3 ml. during the third week. The dogs were bled on the 7th day after the last injection.

Human anti-egg albumin serum.—This serum again was given to us by Professor J. R. Marrack to whom it was sent by Dr. Frankland of St. Mary's Hospital, London. From Dr. Frankland we understand that the serum was obtained in 1950 from a patient who gave a history of urticaria beginning in 1949. Skin tests against the common allergens which included animal scurfs, moulds and pollens were all negative, so too were the following foods—shrimp, fish, onions, mutton, tomatoes and cheese, but he gave a very large immediate positive response to egg albumin.

Guinea-pig anti-horse dandruff protein.—Two sera were tested. The blood was taken after two courses of intraperitoneal injections. each course consisting of four injections of 0·5, 1, 1·5, and 1·5 ml. of a 0·65 per cent solution of horse dandruff protein.

Human sera from persons shown to be sensitive to horse dandruff protein.—These sera were sent to us by Dr. C. J. C. Britton. The sera were from patients giving a positive skin test to horse dandruff protein.

B. Man. (22.ii.52) a patient with asthma who had received no desensitizing injections.

M. Ste. (15.vii.52) a patient with asthma and vasomotor rhinitis. He had received no injections of horse dandruff protein since 1945.

Sug. (20.vi.52)—a patient with asthma and vasomotor rhinitis. He had never received injections of horse dandruff protein.

Puck.—a patient with asthma sensitive to horse, cat and dog dandruff and dust. He had received a very weak course of injections in 1951. First blood sample taken on 1.iv.52. He was then given a course of desensitizing injections and bled again on 3.vii.52.

Fli.—a patient with asthma and vasomotor rhinitis, sensitive to pollen, dust and cat and horse dandruff. He had received no desensitizing injections before 5.iii.52. The blood sample on 3.vii.52 was taken after a course of injections. No further injections were given but another blood sample was taken on 27.x.52.

The other sera tested were from persons who were not suspected of being sensitive to horse dandruff.

Unless otherwise stated all the above sera and the normal control sera were heat-inactivated and absorbed free of antibodies to bovine red cells before being used in any of the tests.

Examination of Sera for Complete or Incomplete Antibodies to Soluble Protein Antigens.

Theoretically the method may be used for any protein antigen, but in the following description egg albumin has been taken as an example. The method may also be adapted to the examination of sera from various animal species.

Examination of guinea-pig serum for complete or incomplete antibodies to egg albumin.

Stage A : Preparation of the egg albumin antigen-bovine cell unit.—A 2 per cent suspension of washed " inagglutinable " bovine red cells (2 ml.) is mixed with an optimal dilution (usually about 1/20) of the rabbit anti-bovine red cell serum conjugated with egg albumin. The mixture is incubated at 37° for 30 min. whereupon the tube is centrifuged and the deposited cells washed twice in saline and finally re-suspended in 2 ml. undiluted heat-inactivated normal rabbit serum, previously absorbed free of antibodies to bovine red cells. The significance of this serum will be discussed later. These cells in suspension constitute the egg albumin antigen-bovine cell unit.

Stage B : Exposure of the egg albumin antigen bovine cell unit to the serum under investigation.—The above suspension in 0·1 ml. amounts is added to 0·1 ml. amounts of increasing dilutions of the heat-inactivated guinea-pig serum being examined for antibodies to egg albumin. After 30 min. at 37° each tube is centrifuged and the cells washed twice in saline and finally re-suspended in 0·1 ml. saline.

In order to see if any guinea-pig antibody globulin has been adsorbed on to the egg albumin antigen-bovine cell unit the cells are subjected to the further procedures which comprise the detector system—for even if the anti-egg albumin antibodies are potentially capable of producing precipitation or agglutination none is observed at this stage of the test owing to the nature of the red cells.

Stage C : Detector system for adsorption of guinea-pig antibodies to egg albumin.—To the individual 0·1 ml. washed cell suspensions is added 0·1 ml. of a dilution (usually 1/20) of rabbit anti-guinea-pig globulin serum. An excess of antibodies to guinea-pig globulin should be added at this stage. After 15 min. at 37° the cells are once again washed twice in saline and re-suspended in 0·1 ml. saline.

Normal inactivated guinea-pig serum, 0·1 ml. of a 1/20 dilution, is then added to each tube in order to saturate the free combining groups of the adsorbed anti-globulin molecules. After 15 min. at 37° the cells are washed twice in saline and again re-suspended in 0·1 ml. saline. One drop from each tube is finally added to small precipitin tubes containing one drop of an optimal dilution (usually 1/40) of rabbit anti-guinea-pig globulin serum. The mixtures are incubated at 37° until the cells have sedimented when each tube is examined for agglutination both macroscopically and microscopically. If guinea-pig antibodies to egg albumin have been adsorbed on the egg albumin antigen-bovine cell unit the building up of the chain of guinea-pig globulin rabbit anti-guinea-pig globulin is able to link the cells together producing agglutination, despite the presence of the hypothetical " prongs " protruding from the surface of the red cells.

Examination of sera for anti-protein antibodies using tanned red cells " coated " with the protein antigen.

The procedure adopted was that described by Boyden (1951). The egg albumin and horse dandruff protein were used as 1 per cent solutions to " coat " the tanned human red cells.

EXPERIMENTS AND RESULTS.

Stable Egg Albumin Antigen Red Cell Unit Capable of Detecting Antibodies to Egg Albumin in Guinea-pig Serum.

The object of this experiment was to see how dilute the rabbit anti-bovine red cell serum conjugated with egg albumin could be used in the preparation of the egg albumin antigen-bovine cell unit.

A 2 per cent suspension of bovine red cells in 0·1 ml. amounts was added to an equal vol. of a series of progressive dilutions of the rabbit anti-bovine cell serum conjugated with egg albumin. After 30 min. incubation at 37° the cells in each tube were washed and exposed to a 1/20 dilution of a guinea-pig anti-egg albumin serum. Stage B and C were then continued as described under ' METHODS.'

Agglutination was observed with the cells exposed originally to the rabbit anti-bovine red cell serum conjugated with egg albumin in dilutions from 1/5 to 1/500. In a comparable test using the rabbit anti-bovine cell serum which had

not previously been conjugated with egg albumin no agglutination was observed. Likewise in another comparable test in which the cell suspension treated with the varying doses of rabbit anti-bovine cell serum conjugated with egg albumin were subsequently exposed to a 1/20 dilution of normal guinea-pig serum instead of a guinea-pig anti-egg albumin serum no agglutination was observed.

It should be mentioned that the " inagglutinable " bovine red cells when sensitized with dilutions of a first course rabbit anti-bovine cell serum of about 1/20 or stronger tend to agglutinate when firmly deposited by high speed centrifugation although on settling by their own sedimentation they remain unagglutinated. The suggestion seems to be that the hypothetical " prongs " which keep the cells apart under normal conditions may be, as it were, bent back by the force of powerful centrifugation thus allowing the antibodies to link across from cell to cell (see Fig. 3). In the present tests the cells are read for agglutination after gently sedimenting of their own accord.

Demonstration of Antibodies to Egg Albumin in Human Serum and the Sera of Guinea-pigs and Dogs using an Egg Albumin Antigen-Bovine Red Cell Unit.

The egg albumin antigen-bovine cell unit was prepared as described in 'METHODS' and was the same whether the serum to be examined for antibodies to egg albumin was from the guinea-pig, dog or man. The conjugated rabbit anti-bovine cell serum was used at a dilution of 1/20.

Each anti-egg albumin serum together with the normal serum controls was examined as in Stage B. The appropriate rabbit anti-globulin sera and normal sera containing globulin were used in the detector systems.

The titre of antibody demonstrated in the sera by this method together with the titre found using the tanned cell method of Boyden is shown in Table I. Normal guinea-pig, dog and human sera gave no demonstrable reaction at a dilution of 1/2 or greater. The finding that the method detected antibodies to practically the same titre as that obtained by the tanned cell method suggests that the method besides being theoretically capable of detecting incomplete anti-protein antibodies when they occur, is also very sensitive.

Demonstration of Antibodies to Horse Dandruff Protein in the Sera of Guinea-pigs and Man using a Horse Dandruff Antigen-Bovine Red Cell Unit.

In a preliminary test it was found that the rabbit anti-bovine cell serum conjugated with horse dandruff protein could be diluted 1/320 and still produce an antigen-cell unit for this system. Using a 1/20 dilution of the conjugated anti-bovine cell serum to form the antigen cell unit, antibodies to horse dandruff protein in two guinea-pig antisera could be demonstrated to a titre of 320. Normal guinea-pig sera gave no reaction at a dilution of 1/2.

Using this system adapted for the detection of human antibodies, the human sera recorded in Table II were examined for antibodies reactive against horse dandruff protein with the results shown. Previous to testing the sera were heat-inactivated for 30 min. at 56° and it should be borne in mind that such a procedure might destroy certain types of antibodies. However it is not intended in this paper to comment in any way on these results ; they are recorded merely as an

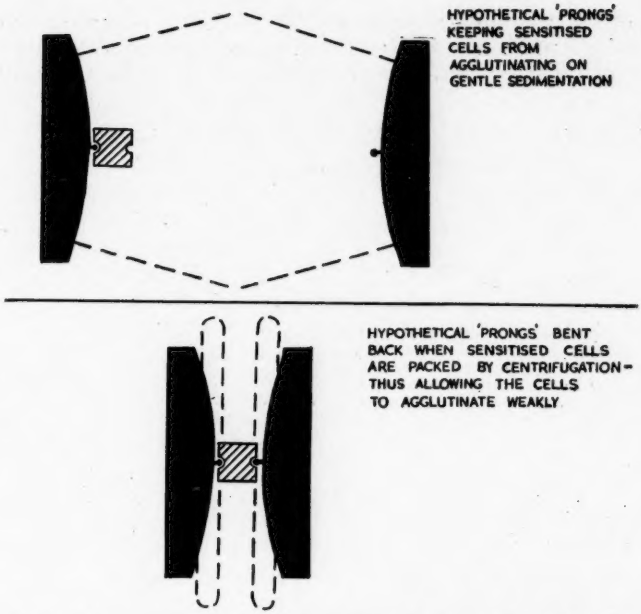
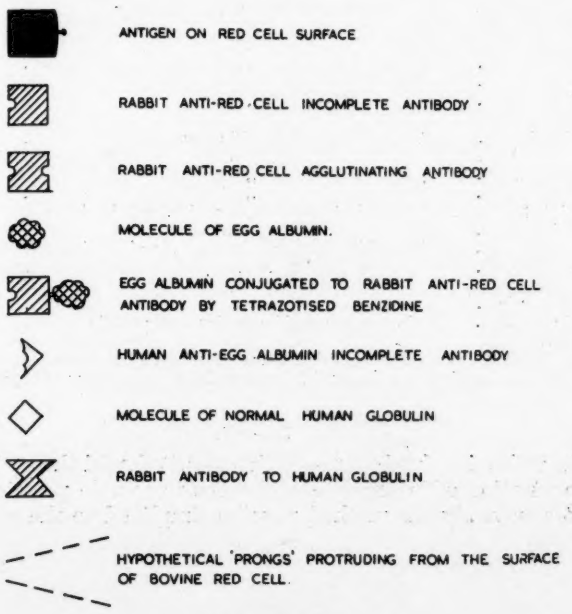

HYPOTHETICAL 'PRONGS' KEEPING SENSITISED CELLS FROM AGGLUTINATING ON GENTLE SEDIMENTATION
HYPOTHETICAL 'PRONGS' BENT BACK WHEN SENSITISED CELLS ARE PACKED BY CENTRIFUGATION - THUS ALLOWING THE CELLS TO AGGLUTINATE WEAKLY

FIG. 3.—Hypothetical explanation as to why "inagglutinable" bovine red cells strongly sensitized with antibody agglutinate weakly when the cells are deposited by powerful centrifugation although they fail to agglutinate on gentle sedimentation.


ANTIGEN ON RED CELL SURFACE
RABBIT ANTI-RED CELL INCOMPLETE ANTIBODY
RABBIT ANTI-RED CELL AGGLUTINATING ANTIBODY
MOLECULE OF EGG ALBUMIN.
EGG ALBUMIN CONJUGATED TO RABBIT ANTI-RED CELL ANTIBODY BY TETRAZOTISED BENZIDINE
HUMAN ANTI-EGG ALBUMIN INCOMPLETE ANTIBODY
MOLECULE OF NORMAL HUMAN GLOBULIN
RABBIT ANTIBODY TO HUMAN GLOBULIN
HYPOTHETICAL 'PRONGS' PROTRUDING FROM THE SURFACE OF BOVINE RED CELL.

Key to Figures.

illustration of the possible applications of the method. Examination of the sera by the tanned cell method of Boyden failed to demonstrate antibodies other than those in the sera of the guinea-pigs inoculated with horse dandruff protein.

TABLE I.—*Titre of Antibodies to Egg Albumin in Various Sera Demonstrated by the New Method.*

Antisera.	Antibody titre found by— Method described in this paper.	Tanned cell method of Boyden.
Normal guinea-pig	0	0
Guinea-pig anti-egg albumin (pool of 6 sera)	>2560	>2560
Normal dog	0	0
Dog M. 1 anti-egg albumin	8	2
Dog M. 2 „ „	8	2
Dog. F. „ „	32	32
Normal human serum	0	0
Human patient sensitive to egg albumin	64	32

0 = <2.

TABLE II.—*Comparison of Two Methods for Detecting Antibody to Horse Dandruff in Supposedly Negative and Positive Sera.*

	Sera.	History (sensitivity to horse dandruff).	Antibody titre to horse dandruff found by— Method described in this paper.	Tanned cells method of Boyden.
1	J	No reason to suspect sensitivity	0	0
	F	Ditto	0	0
	N	„	0	0
	O	„	0	0
	B	„	(W) 1	0
	M	„	(W) 1	0
2	Sug 20/6/52	Positive skin test	8	0
	B. Man 22/2/52	Ditto	2	0
	M. Ste 15/7/52	„	1	0
	Puck 1/4/52	„	1	0
	„ 3/7/52	Positive skin test (after desensitizing injections)	16	0*
	Fli 5/3/52	Positive skin test	2	0
	„ 3/7/52	Positive skin test (after desensitizing injections)	64	0
	„ 27/10/52	Ditto	16	0
3	Normal guinea-pig		<2	<5
	guinea-pig 2265 and 2267	2nd course anti-horse dandruff protein	320	320

(W) 1 = Partial agglutination with the undiluted serum.

* A very weak agglutination was observed in a single tube containing the serum diluted 1/16.

DISCUSSION.

Despite the rather involved nature of this test, it should theoretically allow the *in vitro* demonstration of incomplete antibodies to soluble protein antigens. It is hoped that eventually the method may be simplified to the extent shown in Fig. 1.

More efficient means of conjugating proteins with antibody globulin are being sought. With the object of reducing the amount of protein antigen required a two stage method for conjugation is being attempted. For instance, if chemical A were such that after being coupled to a protein it would combine with chemical B coupled to another protein but would not react with uncombined protein, then the following procedure could be envisaged. Red cells could be sensitized with rabbit incomplete antibody coupled with chemical A. The suspension of sensitized cells after being washed could be exposed to a solution of the desired protein antigen previously coupled with chemical B. By such a procedure none of the protein coupled with chemical B would be wasted by being conjugated to serum proteins other than the anti-red cell antibody, a wastefulness which of course occurs with the present one stage method for conjugation using tetrazotized benzidine. Nevertheless the present system has worked well owing probably to the large number of sites on the bovine cells with which the anti-red cell antibodies combine. The conjugation with tretazotized benzidine is probably not efficient but ample protein molecules appear to be adsorbed on the cell surface to produce ultimately strong agglutination.

The possibility has to be borne in mind that globulin other than antibody globulin may be adsorbed from the heated serum under investigation onto the protein antigen-red cell unit suspended in saline. This appeared to be the case, especially with guinea-pig serum when it was tested undiluted and in dilutions up to 1/5. The factor adsorbed from such a normal serum could well be of the nature of the so-called agglutination enhancing factors (Dean, 1911; Pike, Sulkin and Coggeshall, 1951). This complication however was practically eliminated when the protein antigen-red cell unit was suspended in undiluted heat-inactivated rabbit serum instead of saline. Presumably the affinity of the cell suspensions for substances such as agglutination-enhancing factors was satisfied by the rabbit serum proteins thus blocking any further absorption of such a factor from the guinea-pig or other serum under investigation.

There is a special consideration which has to be borne in mind if it is desired to examine an unheated serum for the presence of antibodies. The protein-antigen-red cell unit would be lysed in the presence of haemolytic complement, and the possibility exists that certain of the antibodies concerned in allergic manifestations may be heat-labile. The sera from persons sensitive to horse dandruff shown in Table II were examined unheated after the complement had been removed by absorption with bovine red cell ghosts sensitized with rabbit antibody. The demonstrable antibody titre however was the same as that found with the heated sera, but again the possibility remains that certain antibodies to horse dandruff, perhaps of the skin-sensitizing type, might have been absorbed non-specifically on the sensitized red cell ghosts. Other methods of removing complement from a serum will have to be investigated for it is now our intention to use this new procedure, or a modification of it, to study the antibodies concerned in different allergic states.

SUMMARY.

A serological procedure is described which theoretically should be capable of detecting incomplete antibodies to soluble protein antigens.

In brief the method involves attaching the protein antigen to the surface of red cells. This is achieved by coupling by chemical means the protein to red cell

antibodies which on combination with the red cells carry the coupled protein antigen to the red cell surface whilst leaving the cells stable in suspension. Adsorption of antibodies to the coupled protein antigen is then detected by means of an anti-globulin serum.

The method has been used experimentally to detect antibodies to egg albumin and horse dandruff protein in guinea-pig, dog and human sera.

The authors wish to express their thanks to Dr. C. J. C. Britton, Professor Grabar, Professor J. R. Marrack and Dr. A. W. Frankland for specimens of serum and to Miss B. D. Disbrey for preparing the figures.

They also wish to express their sincere appreciation to both the Agricultural Research Council and the Nuffield Foundation for a grant in aid of this work.

REFERENCES.

BOYDEN, S. V.—(1951) *J. exp. Med.*, **93**, 107.
COOMBS, R. R. A., GLEESON-WHITE, M. H., AND HALL, J. G.—(1951) *Brit. J. exp. Path.*, **32**, 195.
Idem, HOWARD, A. N., AND WILD, F.—(1952) *Ibid.*, **33**, 390.
DEAN, H. R.—(1911) *Proc. Roy. Soc.* (B), **84**, 416.
PIKE, R. M., SULKIN, S. E., AND COGGESHALL, H. C.—(1951) *J. Immunol.*, **66**, 107.
SQUIRE, J. R.—(1950) *Clin. Sci.*, **9**, 127.

THE EFFECTS OF CORTISONE AND ADRENOCORTICOTROPHIC HORMONE ON DISPERSION OF BRUISES IN THE SKIN.

R. R. H. LOVELL, G. B. D. SCOTT, B. HUDSON* AND J. A. OSBORNE.*

From the Rheumatism Research Unit of the Department of Medicine and from the Department of Pathology, St. Mary's Hospital Medical School, London.

Received for publication April 29, 1953.

CUSHING (1932) described haemorrhages in the skin as a feature of the syndrome which bears his name. Apparently spontaneous bruises also occur in some patients receiving cortisone and adrenocorticotrophic hormone (ACTH). When we first saw these lesions in two patients, one receiving cortisone and the other ACTH, we thought the discoloration persisted for an unusually long time; bruises produced by venipuncture also disappeared very slowly. These observations led us to study the effects of cortisone and ACTH on the dispersion of bruises induced experimentally in the skin.

We first describe studies in man, which confirmed our original observation; the hormones delayed dispersion of experimental bruises and delay also occurred in Cushing's syndrome. Since a detailed understanding of this effect depended upon histological examination of tissues, we also investigated bruise dispersion in the guinea-pig and the effect of cortisone on it.

Dispersion of Bruises in Man.

Experimental bruises were made in healthy subjects and in patients with rheumatoid arthritis and other diseases. With a tuberculin syringe and gauge 26 needle, 0·1 ml. of the subject's own blood was injected intradermally. The blood was withdrawn from an antecubital vein and injected before clotting occurred. Duplicate injections were made, in some subjects in the flexor surfaces of the forearm, in others into the skin of the back. When treatment effects were studied, bruises were made at approximately weekly intervals and care was taken to use comparable sites before, during and after treatment. Bruises were inspected daily until they disappeared.

The lesions had three components 24 hr. after injection. In the centre was an area 2–3 mm. diameter, which was slightly reddened, raised and indurated. Around this was a mauve coloured ring 8–12 mm. in diameter, with clearly defined margins. Surrounding this was an area of staining 15–20 mm. diameter, whose margins were less distinct and whose tint varied, being usually yellow or brown. Later the central area became pale and the peripheral area developed hues of blue and green which faded imperceptibly into normal skin. The mauve zone became interrupted by pale patches and it was possible to determine with fair precision the day on which the last trace of mauve disappeared. The time taken for the disappearance of the mauve was used as the index of dispersion and the mean dispersion time in days of the duplicate lesions was recorded.

* Holders of Nuffield Foundation Fellowships.

Dispersion Times in Untreated Subjects.

The dispersion times measured on one occasion in each of three groups of subjects are recorded in Table I. The range of dispersion times is wide but similar in each group. The mean dispersion times are also similar and a t test showed no significant differences between groups. Although this would not necessarily be true if a larger number of observations were analysed, it is clear that the difference between the three kinds of subject tested is unlikely to be great.

The constancy of dispersion times over several weeks was also tested. Table II shows the results in two patients tested at intervals of one or two weeks. In both cases the times are fairly constant and fall within one day of the means.

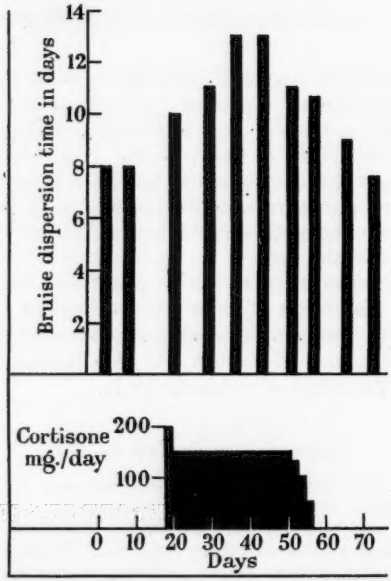


FIG. 1.—Sequence of bruise dispersion times related to treatment in Case 3.

Effect of Cortisone and ACTH on Dispersion Times.

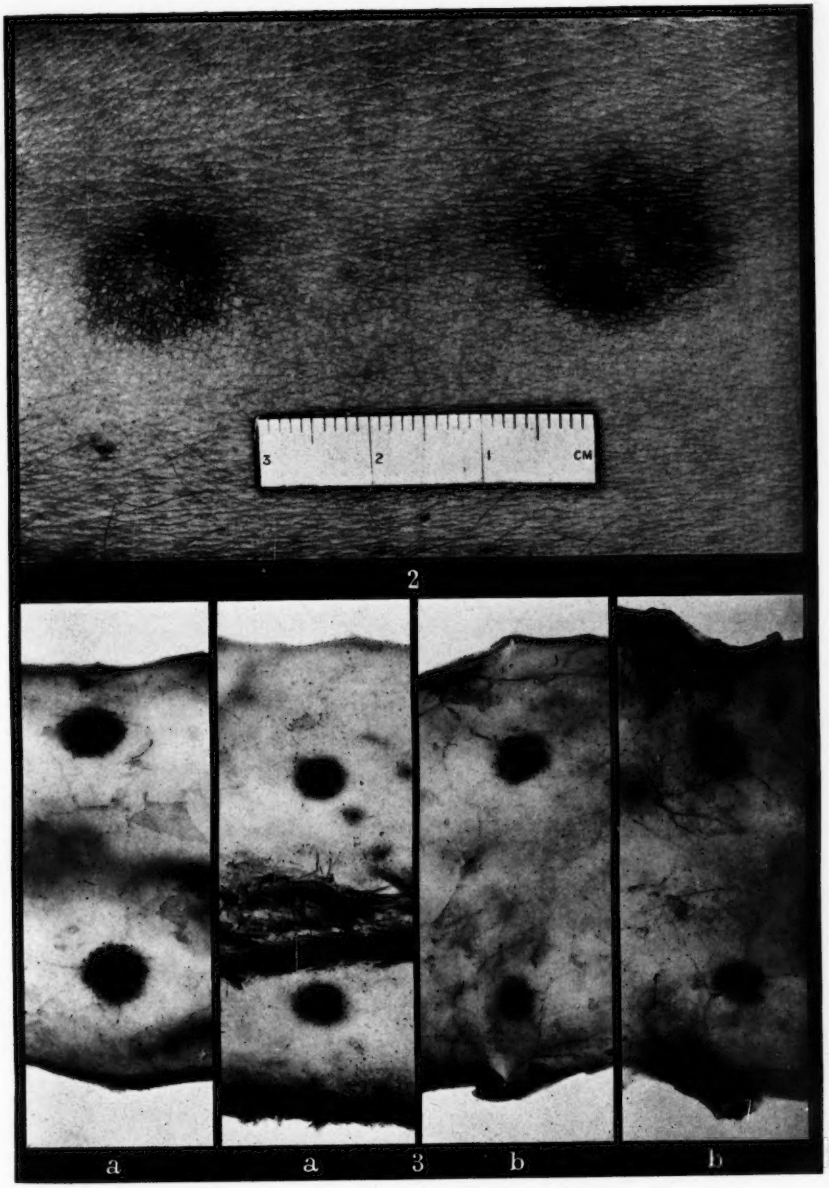
This was studied in 8 patients, 1 of whom had ulcerative colitis and the others chronic active rhematoid arthritis. Table III summarises the results, and the sequence of dispersion times in case 3 is illustrated diagramatically in Fig. 1.

Patients 1 and 2 were those in whom the original observation of persistent staining in spontaneous bruises and at venipuncture sites were made. In both, experimental bruises were made in the last week of treatment and compared with

DESCRIPTION OF PLATES.

FIG. 2.—Appearance of 7-day-old experimental bruises in the skin of the forearm during ACTH treatment in patient 2.

FIG. 3.—Transilluminated skins in experiment 2. Three-day-old experimental bruises (*a*) in guinea-pigs treated with cortisone. (*b*) in guinea-pigs treated with diluent only. × 1.



Lovell, Scott, Hudson and Osborne.

bruises made after treatment was stopped. Dispersion times were prolonged during, as compared with after, treatment by 2·5 and 8·0 days respectively. The site of 7 day-old lesions during ACTH in patient 2 is shown in Fig. 2. In patients 3, 4, 5, 6 and 7 prolongation of dispersion times during, as compared with before, treatment ranged from averages of 1·8 to 7·5 days. In three of the first four, the difference was statistically significant by the *t* test.

Prolongation of dispersion time was not always attained in the first 2 weeks of treatment, in cases in which it was eventually increased ; this may account for the absence of change in patient 8 who received cortisone for only 2 weeks. Prolonged dispersion time may not, however, invariably accompany treatment.

TABLE I.—*Bruise Dispersion Times in Healthy and Diseased Subjects.*

Group.	Number of subjects.	Bruise dispersion time in days.		
		Mean.	S.D.	Range.
1. Healthy . . .	10	6·5	1·7	4–9
2. Rheumatoid arthritis .	10	6·2	1·1	4–8
3. Other diseases . .	8	6·8	1·3	5–9

TABLE II.—*Bruise Dispersion Times in Repeated Tests in 2 Patients.*

Diagnosis.	Tests.				
	1.	2.	3.	4.	5.
Inactive choroiditis .	7·5	8·5	9	8·5	8·5
Rheumatoid arthritis .	6·0	5·0	6·0	6·0	

The tests were made at intervals of 1–2 weeks.

TABLE III.—*Effect of Cortisone and ACTH on Bruise Dispersion Times.*

Patient.	Age.	Sex.	Diagnosis.	Daily dose (mg.).	Days.*	Mean bruise dispersion time in days.				
						Before.	During.	Increase.	S.E. of difference.	P.	After.
1	44	F.	Rheumatoid arthritis	Cortisone 100–150	50		9·5 (1)				7·0 (1)
2	53	M.	Rheumatoid arthritis	ACTH. 50	51		14·0 (1)				6·0 (1)
3	39	M.	Ulcerative colitis	Cortisone 150	40	8·0 (2)	11·4 (6)	3·4	0·82	$0·001 < P < 0·01$	8·3 (2)
4	34	M.	Rheumatoid arthritis	Cortisone 150	38	7·5 (2)	9·3 (5)	1·8	0·68	$0·02 < P < 0·05$	8·8 (6)
5	32	M.	Rheumatoid arthritis	ACTH. 80	49	5·8 (4)	9·9 (7)	4·1	0·61	$P < 0·001$	7·4 (7)
6	52	M.	Rheumatoid arthritis	ACTH. 150	14	6·7 (3)	10·3 (3)	3·6	1·34	$0·05 < P < 0·1$	7·0 (2)
7	47	M.	Rheumatoid arthritis	Cortisone 200	14	7·0 (1)	14·5 (1)	7·5			8·0 (1)
8	16	F.	Rheumatoid arthritis	Cortisone 200	14	6·3 (2)	6·5 (1)	0·2			6·5 (1)

* Duration of treatment with cortisone or ACTH before production of bruise.

The figures in brackets are the numbers of pairs of tests on which the times are based.

The latter part of treatment in patients 4 and 6 was supplemented by 1 g. ascorbic acid daily, for reasons not concerned with this experiment. There was no change in dispersion time attributable to this supplement.

After treatment, bruise dispersion was accelerated. In patients receiving more than 2 week's treatment, the dispersion time decreased gradually and sometimes irregularly over several weeks and the mean dispersion times in the after-treatment periods were thus greater than those before treatment.

Bruise Dispersion Time in Cushing's Syndrome.

Because cortisone and ACTH administration may induce all the manifestations of Cushing's syndrome, experimental bruises were studied in three such cases. All were females ; one had an adrenal cortical carcinoma and the others adrenal cortical hyperplasia. The bruise dispersion times were : over 12, 12·5 and 11·5 days respectively. These exceed the range of dispersion times of 4–9 days found in subjects without Cushing's syndrome and are similar to times found in some patients treated with cortisone and ACTH (Table III).

Dispersion of Bruises in Guinea-pigs.

Albino guinea-pigs of the Compton strain were used. They weighed 400–500 g. and were fed on Bruce and Parkes (1947) pellet diet and unrestricted cabbage. Treatment consisted either of cortisone acetate (Cortone, Merck) or the diluent alone. Cortisone was injected subcutaneously 10 mg. daily, and the same volume of diluent 0·4 ml. was injected in control animals.

Bruises were made by duplicate intradermal injections of 0·1 ml. fresh heart blood in the skin of the closely clipped back, one on each side of the midline.

Experiment 1.—Two injections of blood, obtained from a single donor, were made in each of four female guinea-pigs, in order to discover the dispersion time of the bruises.

Experiment 2.—To study the effects of cortisone on bruise dispersion, four male guinea-pigs were used, 2 receiving cortisone daily and 2 the diluent, for 10 days before death. Their own blood was injected 7, 3 and 1 days before death, and the lesions were examined macroscopically and histologically.

Experiment 3.—The macroscopic and histological study was extended, using 8 females, 4 receiving cortisone treatment for 7 days before death. Their own blood was injected 4, 3 and 2 days before death. One cortisone-treated animal died under anaesthesia during the experiment.

The lesions for histological examination were excised from the freshly killed animals, fixed in 10 per cent formol saline, and sections were stained with haematoxylin and eosin, haematoxylin and van Giesen and a combination of Perl's and Turnbull's methods for iron, using a mixture of 2 per cent potassium ferrocyanide and 2 per cent potassium ferricyanide with 1 per cent Hydrochloric acid.

Macroscopic Appearances.

The appearances of the bruises were similar to those in man. In all experiments the bruise dispersion time in untreated animals was 4 days.

The 1- and 2-day-old lesions were indistinguishable in the cortisone-treated and control animals. At 3 days the bruises in the former were still compact and homogeneous, while in the latter disruption of mauve pigment was taking place (Fig. 3). Differences between the groups, seen best by transillumination of the skin, were even more marked at 4 days, when lesions in the test group were smaller but still compact, whereas in the control group they appeared as pale areas with ill-defined margins.

Histological Appearances.

There was conspicuous polymorphonuclear infiltration at the centre of the 1- and 2-day-old lesion from treated and control animals. This was reduced by 3 days and almost absent by 4 days.

The injected erythrocytes lay at first between the collagen bundles of the corium and in the adipose tissue deep to it. In the test animals they were still present at 4 days, mostly in the adipose tissue layer, but they were absent by this time in 3 out of the 4 control animals; in the fourth animal, the residual erythrocytes appeared fewer than in the corresponding lesions in the test group. In a 4-day-old lesion in one of the test group, erythrocytes extended from the adipose layer deep to the corium into the underlying muscle layer,where they lay free between the muscle bundles. In all the lesions the erythrocyte aggregations corresponded in position with the mauve pigment zone seen macroscopically.

In the 1- and 2-day-old lesions, some of the histiocytes had enlarged and by 3 days they had greatly increased in number. The histiocyte proliferation occurred evenly throughout the corium and did not appear to be concentrated round the more patchily distributed erythrocyte aggregations. Histiocytes were still conspicuous at 7 days, 3 or 4 days after disappearance of the erythrocytes. No definite differences in histiocyte reaction were established between the treated and control groups.

Erythrophagocytosis by histiocytes, although present from the first day, was inconspicuous. An examination was made of 200 histiocytes in the corium and 100 in the adipose layer in 2-, 3- and 4-day-old lesions from both treated and control animals. Table IV shows that the percentage of histiocytes showing erythrophagocytosis was very small and did not differ appreciably in the two groups. More than one erythrocyte was rarely seen in any one histiocyte.

Local haemosiderin formation was very scanty and was not seen until the third day, when it appeared almost confined to the adipose layer deep to the corium. At 7 days, some brown crystals were seen which did not give the staining reaction for iron and which were thought to be haematoidin.

TABLE IV.—*Percentage of Histiocytes showing Injected Erythrocytes in Experimental Bruises in Guinea-pigs.*

Age of lesion in days.	Site.	Per cent histiocytes with ingested erythrocytes.	
		Cortisone group.	Control group.
2	Corium	1·0	2·0
	Adipose layer	2·0	2·0
3	Corium	0·0	3·0
	Adipose layer	0·0	1·0
4	Corium	1·0	1·0
	Adipose layer	0·0	0·0

DISCUSSION.

We have shown that dispersion of skin bruises is delayed by cortisone, ACTH and in Cushing's syndrome in man and by cortisone in guinea-pigs. We have not succeeded in identifying with certainty the mechanism or mechanisms by which this hormone effect is attained, but we would make the following comments.

The factors concerned in dispersion of extravasated erythrocytes are complex and incompletely studied in any one species or tissue. Cohnheim (1889) described removal by lymphatics, decolourization with remnants of colourless stroma,and ingestion by phagocytes. When we first saw delayed bruise dispersion in man, we suspected that it was due to an impaired macrophage response, for Gell and Hinde (1951) had found that this response was impaired in inflammatory reactions in rabbits treated with cortisone and ACTH. However, in 2- to 6- day-old experimental bruises which we excised from untreated patients, phagocytosis of erythrocytes was so inconspicuous that it seemed unlikely that an impaired macrophage response, at least alone, could account for the delayed bruise dispersion. Our doubts about this were confirmed by the studies in guinea-pigs which we have described; again, removal of erythrocytes by phagocytes appeared to play a minor part in bruise dispersion.

The relatively minor phagocytosis seen in our lesions contrasts with that described by Muir and Niven (1935). This might be partly due to differences in the methods employed in preparing tissues for examination, for Muir and Niven's striking illustrations were of " spread " preparations, a technique hardly applicable to the tough skin of the guinea-pig. The different findings are more likely to be related to the differences in volume of blood injected, the sites of injection and the species studied. We injected intradermally smaller volumes than Muir and Niven injected subcutaneously, and our lesions dispersed more rapidly. Muir and Niven studied the mouse, rat and rabbit, while we studied man and the guinea-pig. It is of interest too that they found erythrophagocytosis less conspicuous in the rabbit than in the mouse and rat.

As a result of our experiments, we have been led to consider other possible means whereby cortisone might delay bruise dispersion. The peripheral staining with haemoglobin and its derivatives showed that haemolysis occurred in our experimental lesions. Though it is conceivable that cortisone delays bruise dispersion by influencing the rate of haemolysis, this seems unlikely, for we saw no difference in the development of staining in treated and untreated subjects.

To try to assess the part played by lymphatic removal of erythrocytes, lesions were excised with surrounding skin, cleared and examined microscopically. Though small blood vessels were shown up by the blood they contained, lymphatic trunks were not detected. We cannot therefore judge the part played by lymphatics in removing erythrocytes from these lesions or the possible effect of cortisone on lymphatic removal.

Another mechanism of bruise dispersion is the diffusion of erythrocytes through the surrounding tissues. In microscopic sections through our experimental bruises, the erythrocytes lay between the collagen bundles, in spaces which contain ground substance (Gersh and Catchpole, 1949), between fat cells and, deeper, between muscle fibres. Erythrocyte dispersion might be influenced by factors affecting the resistance offered by ground substance to cell movement. Hindrance to cell movement by altered ground substance has also been considered by Gell and Hinde (1953) as an explanation of the impaired macrophage response which they found in inflammations in rabbits treated with cortisone. The possibility that cortisone and ACTH influence bruise dispersion by affecting the quality of ground substance clearly requires further study. In this connection we would observe that the presence of free fluid in the tissues, such as occurs in inflammation, is one of the factors which enhances the diffusion of substances injected in the

skin (Seeberg, 1947). There was an inflammatory reaction in our experimental bruises, and the well-known effect of cortisone in diminishing inflammations in the skin of animals, which we have also shown to occur in man (Lovell, Goodman, Hudson, Armitage and Pickering, 1953), may possibly be relevant to the effect of cortisone on bruises.

SUMMARY.

In man, the dispersion of bruises in the skin is delayed by treatment with cortisone and ACTH and in Cushing's syndrome. In guinea-pigs it is delayed by cortisone.

The results of histological studies make it unlikely that the effect can be attributed to impairment of macrophage activity. The effect may be due to impaired lymphatic removal of erythrocytes or to altered resistance to their dispersion through the ground substance of connective tissue.

We are indebted to the Nuffield Foundation for the grant which enabled these studies to be undertaken and to Merck and Co. Inc., part of whose gift of cortisone to the joint committee of the Nuffield Foundation and the Medical Research Council was used. We are grateful to Dr. I. T. Hinde and Dr. D. Pike for commenting on some of the histological preparations and to Dr. P. N. Cardew for the photographs. We thank Professors W. D. Newcomb and G. W. Pickering for their encouragement.

REFERENCES.

Bruce, H. M., and Parkes, A. S.—(1947) *J. Hyg. Camb.*, **45**, 70.
Cohnheim, J.—(1889) 'Lectures in General Pathology.' London (New Sydenham Society). (Vol. I, 406.)
Cushing, H.—(1932) *Johns Hopk. Hosp. Bull.*, **50**, 137.
Gell, P. G. H., and Hinde, I. T.—(1951) *Brit. J. exp. Path.*, **32**, 516.—(1953) *Ibid.*, **34**, 273.
Gersh, I., and Catchpole, H. R.—(1949) *Amer. J. Anat.*, **85**, 457.
Lovell, R. R. H., Goodman, H. C., Hudson, B., Armitage, P., and Pickering, G. W.—(1953) *Clin. Sci.*, **12**, 41.
Muir, R., and Niven, J. S. F.—(1935) *J. Path. Bact.*, **41**, 183.
Seeberg, G.—(1947) *Acta derm.venereol. Stockh.* (Suppl. 18, 1.)

THE FRACTIONATION OF LIPIDS WHICH INHIBIT HAEMAGGLUTINATION.

E. C. CLARK, G. H. LATHE AND C. R. J. RUTHVEN.

From the Bernhard Baron Memorial Research Laboratories, Queen Charlotte's Maternity Hospital and the Institute of Obstetrics and Gynaecology, University of London, London, W.6.

Received for publication April 23, 1953.

LIPID extracts of tissues will prevent the agglutination of red blood cells by appropriate antisera. Murray, Lathe, Clark, Ruthven and Levine (1950) showed that this property of lipid extracts was not specific for one blood group system. They made a preliminary fractionation of lipid extracts by adsorption chromatography and produced three inhibitory materials, all of which were considered to be mixtures. Further attempts to purify these materials by adsorption chromatography were unsuccessful but eventually a more effective method of lipid fractionation by counter-current distribution (Cole, Lathe and Ruthven, 1953*a*, *b*) was devised. This method has now been applied to the separation of lipids inhibiting haemagglutination.

METHODS.

Murray *et al.* (1950) have described the extraction of tissue lipids, the preparation of inhibitors, and serological techniques for determining the inhibitory activity. These latter consisted essentially of dispersing a definite quantity of lipid in a diluted agglutinating antiserum (anti-D) which was then added to a suspension of Rh-positive cells in pH 7·34 phosphate buffer. The extent of inhibition of agglutination was read at 15, 60 and 120 min., comparisons being made with appropriate controls.

Human placenta and brain, from which the membranes had been removed, were dehydrated by homogenizing twice with two vol. of acetone. The remaining lipids were then extracted by homogenizing the tissue residue with about twice the original vol. of alcohol-ether (2 : 1), and allowing the material to stand for several hours. The acetone and alcohol-ether extracts were taken to dryness *in vacuo* at $< 40°$ and the lipid residues combined. Samples of lipid (0·5–2·0 g.) were then fractionated by dissolving them in equal vol. (usually 20 ml.) of the two phases of solvent system A (Table I) and distributing them through 49 tubes in the counter-current apparatus of Lathe and Ruthven (1951). The lipid in each tube was recovered and estimated by evaporating the solvent at $< 40°$ *in vacuo*, drying over H_2SO_4 *in vacuo*, and weighing the residue. The amount in individual tubes is recorded as a percentage of the whole by wt. Further distributions were made with solvent systems B, C, and D (Table I) depending on the polarity of the lipid.

EXPERIMENTAL.

Counter-Current Distribution of Inhibitors.

Previously Murray *et al.* (1950) fractionated lipid extracts by adsorption chromatography on alumina, and found that some inhibitory activity occurred in the light petroleum eluate (inhibitor " P "), and in a methanol eluate (inhibitor " M "), while further inhibitor material (inhibitor " R ") could be washed from the column with hot methanol and ether. These materials provided the starting point for counter-current studies. A sample of each of the inhibitors was subjected

TABLE I.—*Composition of solvent systems for counter-current distributions.*

	System.			
	A	B	C	D
Volume ratio of upper to lower phase	1/1	1/4	1/1	1/1
Components	CCl_4 62	As	L.P. 31	CCl_4 25
	CH_3OH 35	System	CCl_4 31	$CHCl_3$ 15
	H_2O 3·15	A	CH_3OH 35	CH_2Cl_2 10
			H_2O 3	CH_3OH 40
				H_2O 10

Figures in the body of the table are volumes of individual components required to prepare a system; L.P. = light petroleum, b.p. 40–60°.

to 49 transfers with solvent A. The results of these distributions (Fig. 1). showed that each of the inhibitors was a mixture of many different fractions. For this reason it seemed appropriate to determine the position occupied by inhibitory materials in counter-current distributions of crude placenta lipid.

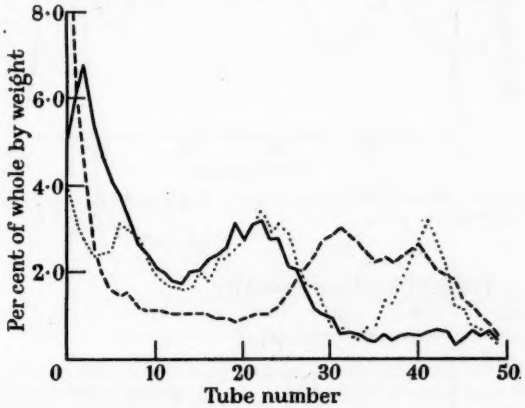


FIG. 1.—Counter-current distributions of Inhibitor M (.....), Inhibitor P (———), and Inhibitor R (– – – –) through 49 tubes in solvent A.

Inhibitors in distributions of crude placenta lipid.

A placenta lipid extract was distributed through 49 tubes in solvent A (Fig. 2) and the material in every second tube was examined for inhibitory activity, using 0·1 mg. of lipid. By studying a series of tubes at one time it was found possible to pick out the "peaks" of activity without difficulty. Three peaks were observed in the region of tubes 4, 18 and 24, and there was no inhibitory activity in tubes 30–49.

For more complete separation the polar fractions (tubes 0–16) were combined and distributed in solvent D (Fig. 3). Strong inhibitory activity was found between tubes 2 and 26. Accordingly these tubes were reassayed using 0·05 mg. of lipid. This revealed a main peak of inhibition at tube 20, and a minor one at tube 4. It was observed that fractions comparable with tube 20 tended to be

lytic when assayed at the usual 0·1 mg. level. When examined under the microscope this lytic process consisted of an explosive disruption of the erythrocyte membrane, as is seen in lysis by bile salts. This provided a contrast with lysis by anionic detergents in which there is a gradual escape of the haemoglobin through the membrane.

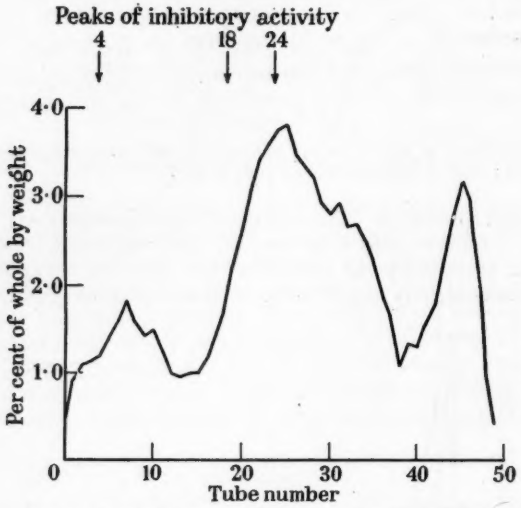


FIG. 2.—Position of the peaks of inhibitory activity in a 49-tube distribution of crude placenta lipid in solvent A.

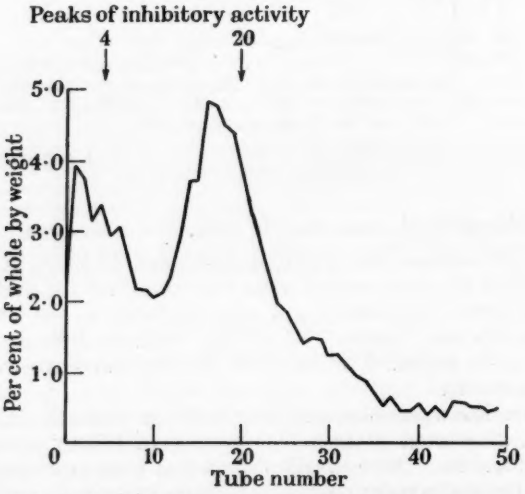


FIG. 3.—Position of the peaks of inhibitory activity in a 49-tube distribution in solvent D of the combined placenta lipid fractions from tubes 0–16 (see Fig. 2).

The central fractions (tubes 17–38) of the first distribution of placenta lipid (Fig. 2) were further examined. They were combined and after a preliminary division by a 9-tube distribution in solvent A the more polar tubes (0–4) and the less polar tubes (5–9) were combined and distributed separately through 49 tubes in solvent C (Fig. 4). A strong inhibitor was found in both distributions in tube 2, a weaker one in tube 17, and a third, scarcely inhibitory, in tube 25. The lack of activity in tubes 30–49 of the first distribution (Fig. 2) was confirmed by running these lipids in another solvent system and re-examining them. No activity was found.

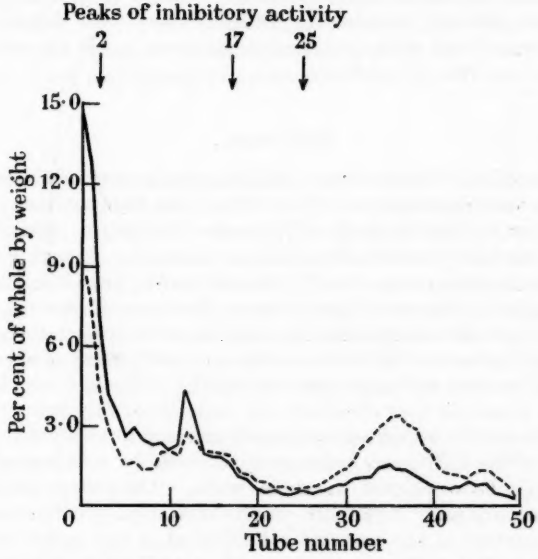


FIG. 4.—Position of the peaks of inhibitory activity in a 49-tube distribution in solvent C of the more polar (———) and less polar (– – – –) parts of the placenta lipid from tubes 17–38 (see Fig. 2).

In addition to examining these distributions of placenta lipid, assays have been made on the fractions of other counter-current distributions of placenta lipids. The results are not presented in detail here, since they merely confirmed the main features which have emerged above.

Inhibitors from brain lipid.

While no systematic search for lipid inhibitors of haemagglutination has been made in brain extracts, observations have been made on the counter-current distributions of cephalin, mixed sphingomyelin and cerebrosides, and lecithin, as prepared from brain (Cole *et al.*, 1953*a*). After preliminary separation in solvent A the polar parts were distributed in solvent D under the same conditions as demonstrated in Fig. 3. Cephalin showed inhibitory peaks at tubes 3–4 and tubes 17–18. In a preparation of mixed sphingomyelin and cerebrosides, simi-

larly treated, inhibitory peaks were found at these same two points. A preparation of brain lecithin was distributed in solvent B, and presented a slightly different pattern of inhibitory lipids. These were found between tubes 3 and 14, the most active being at tube 8 (Fig. 3, Cole *et. al.*, 1953*a*). This lack of agreement with the other brain lipid distributions is probably due to the differences in the two solvent systems.

Brain lipids obtained from tubes 18 to 40 of a 49-tube distribution in solvent A (*cf.* Fig. 2) were re-distributed, as with placenta lipid, in solvent C (*cf.* Fig.4). Strongly inhibiting material was found in tubes 0 and 2, and less so in tube 4, but the two minor inhibitors, corresponding to tubes 17 and 25 of the placenta distribution were scarcely detectable. Possibly they were diluted out by other materials. As was found with comparable placenta lipids the non-polar half of this distribution was free of inhibitors.

DISCUSSION.

These studies of the distribution of haemagglutination inhibitors among lipid fractions permit two observations. It is noted that the position of the peaks of activity is similar in distributions of placenta and brain lipids. Each of the tissues appears to have three main inhibitor fractions, occurring in comparable places in the distributions (tubes 4 and 20 in solvent D ; tube 2 in solvent C). This speaks for a degree of chemical specificity. However, if the activity depended on two or three specific compounds it would be anticipated that as these were freed of inactive materials by counter-current distribution the same inhibitory power would be shown by much less material. This has not been observed. The most active materials now obtained (*e.g.*, tube 20 of Fig. 2) are not much more potent than inhibitor P, which was the most active previously found.

This failure of the inhibitory potency to increase by a factor of three or five, at the least, may be explained in three ways. The active material may be unstable and decomposing under the conditions used for fractionating lipids. Secondly the reduction of the amount of lipid used in the assays may vitiate the conditions of the test, by altering the proportion of lipid to serum protein in the system. Thirdly, the lipid fractions being separated from the main inhibitors may also be inhibitors of agglutination, but with an activity a little lower than that necessary to show up under the conditions of the test. This latter possibility is in keeping with the presence of at least three relatively strong inhibitor fractions, as well as other which are less active. Moreover, the distribution of inhibitory material may be widened considerably by using 0·1 or 0·5 mg. of each fraction for assay. It would be expected then that the inhibitory characteristic was related to some structural feature, or property, common to all these active fractions.

The position of the inhibitors in the counter-current distributions gives some indication of their chemical nature. It is observed that all the active lipids are polar in character, and that the fractions containing neutral fat and cholesterol esters (Fig. 2, tube 45), as well as cholesterol (Fig. 2, tube 35) are devoid of activity. Elsewhere (Cole *et al.*, 1953*a*, *b*) evidence is presented to show that a large part of the polar fractions of brain lipid is phospholipid in character, and this is also true of placenta lipid, though to a lesser extent. The placenta lipid distributions have been examined to see whether the inhibitory activity of lipid fractions could be correlated with the presence of any one phosphatidyl base. Although the

relationship is not a simple one, an examination of the materials from another scheme of fractionation in which the distribution of bases has been more thoroughly worked out suggested that serine occurs in all the strong inhibitors. However not all the serine-containing fractions are strongly inhibitory.

Among lipids, phospholipids are notable for the association of strong hydrophilic and aliphatic characteristics, and it is this combination of opposite qualities to which their surface activity may be attributed. The effect of these substances on haemagglutinating systems may be due to this surface activity. In our experience other surface-acting agents, such as anionic detergents and bile salts, also inhibit agglutination. Further studies are required to show how inhibition is effected, although Murray *et al.* (1950) have shown that the lipid inhibitors do not interfere with the attachment of the antibody to the erythrocyte, but intervene at a later stage in the agglutination process.

SUMMARY.

A search for inhibitors of haemagglutination has been made among fractions of brain and placenta lipid in counter-current distributions. It was observed that the inhibitory property is absent from non-polar lipids, and widespread among polar lipids. The location of the most active materials in distributions of brain lipid is similar to that of placenta. This suggests some chemical specificity. However, the more widespread occurrence of activity when inhibition assays are made with larger amounts of material, together with the absence of any fractions with much greater potency than the cruder mixtures, suggests that some common property of polar lipids, such as surface activity, underlies their inhibitory property.

Our thanks are due to Dr. John Murray who suggested this investigation and gave it his support, and to Miss R. Persky and Mrs. P. G. Cole who assisted in the preparation of materials.

REFERENCES.

Cole, P. G., Lathe, G. H., and Ruthven, C. R. J.—(1953*a*) *Biochem. J.*, **54**, 449.—(1953*b*) *Ibid.*, **55**, 17.

Lathe, G. H., and Ruthven, C. R. J.—(1951) *Ibid.*, **49**, 540.

Murray, J., Lathe, G. H., Clark, E. C., Ruthven, C. R. J., and Levine, M.—(1950) *Brit. J. exp. Path.*, **31**, 566.

THE EFFECT OF STAPHYLOCOCCAL FILTRATES ON ISOLATED RABBIT SMALL INTESTINE, WITH SPECIAL REFERENCE TO ENTEROTOXIC STRAINS OF *STAPH. PYOGENES.*

K. ANDERSON.

*From the Department of Bacteriology, Guy's Hospital Medical School, London, S.E.*1.

Received for publication May 29, 1953.

THE ability of certain strains of staphylococci to produce substances capable of irritating the gastro-intestinal tract has been recognised since the 19th century. Denys (1894) first described the phenomenon in Belgium, while Owen (1907), Barber (1914) and Dack, Cary, Woolpert and Wiggers (1930) all record similar outbreaks. Following the description by Dack and his colleague staphylococcal food-poisoning became an established syndrome and since then many workers have sought to describe tests for the recognition of enterotoxic strains. Jordan and McBroom (1931) used *M. rhesus* monkeys to test toxic filtrates, but results with this animal have been variable. Borthwick (1933) injected filtrates into the stomachs of rabbits and guinea-pigs at controlled pH values, while Stone (1935) has described a gelatin liquefaction test for the recognition of food-poisoning strains.

The intra-peritoneal kitten test of Dolman, Wilson and Cockcroft (1936) attracted considerable attention and for some years it was regarded as a reliable method of identification. However, Fulton (1943) has shown that kitten-positive strains may be harmless to susceptible human volunteers whilst strains which produce characteristic symptoms in volunteers fail to give a positive kitten test. Little progress has been made since that date, and as Van Heyningen (1950) states: "Progress in research on this toxin is stifled almost to the point of extinction by the difficulties of testing the toxin."

The effect of staphylococcal filtrates on isolated small intestine was first investigated by Bayliss (1940) who failed to observe any direct action of the toxin on isolated rabbit and cat small intestine. However, Richmond, Reed, Shaughnessy and Michael (1942) showed that filtrates from a clinically positive strain could produce an increase in tonus in almost all experiments when tested on isolated segments of rabbit jejunum. In some instances this was accompanied by an increase in amplitude of contraction. Richmond *et al.* (1942) concluded that this increased tonicity might be equivalent to the enterospasm experienced in human cases, and that the test could prove useful in detecting enterotoxin production. In view of these conflicting results, it was decided to investigate the effect of various staphylococcal filtrates on isolated rabbit small intestine to decide whether the method was of any value in the identification of enterotoxic strains.

MATERIALS AND METHODS.

Selection of strains.

Twenty-four strains of *Staphylococcus pyogenes* were selected for testing. Twelve of these were isolated from a variety of pyogenic lesions in man, and twelve, which were suspected food-poisoning strains, were kindly supplied by Dr. R. E. O. Williams. All strains were screened for haemolysin production by the plate method described by Elek and Levy (1950).

Preparation of filtrates.

Toxic filtrates were prepared by the method of Dolman and Wilson (1938). Owing to the great reduction of haemolytic activity following sterilization by a Seitz EK disc, the crude culture filtrates were centrifuged at 7000 r.p.m. for 20 min. and the clear supernatant fluids were used immediately without further treatment or storage. The α-haemolysin titre was determined in each case before testing, by incubating filtrates at 37° with a 1 per cent suspension of washed rabbits' red cells. Readings were taken at 1 hr., and control racks with added commercial staphylococcus antitoxin were set up in each instance. The results of these preliminary investigations are found in Tables I and II. Control filtrates were prepared by expressing fluid from uninoculated media.

TABLE I.—*Haemolytic Activity of* 12 *Pyogenic Strains of* Staph. pyogenes.

Strain.	Source.	Haemolysins.	α titre of filtrate.
Ellis	Nose	α δ	1 : 512
Kilda	Meningitis	α δ	1 : 128
R.K.	Boil	α β δ	1 : 256
K.M.A.	Osteomyelitis	α δ	1 : 128
K.A.	,,	α	1 : 16
Mant I	Pneumonia	α δ	1 : 128
Mant II	Pemphigus	α δ	1 : 64
Clarke	Meningitis	α	1 : 4
Johnson	Sinusitis	α δ	1 : 128
Hillman	Carbuncle	α β δ	1 : 512
J.K.	Cellulitis	α δ	1 : 64
Starr	Meningitis	*Nil*	—

TABLE II.—*Haemolytic Activity of* 12 *Food-poisoning Strains of* Staph. pyogenes.

Strain.	Haemolysins.	α titre of filtrate.
50/3003	α δ	1 : 32
50/3147	α δ	1 : 512
50/3400	α δ	1 : 128
50/3529	α δ	1 : 128
51/957	α δ	1 : 128
51/1123	α δ	1 : 64
51/2002	α δ	1 : 256
51/2312	α δ	1 : 128
51/2448	α δ	1 : 128
51/2533	α δ	1 : 64
51/2772	α δ	1 : 128
51/3100	α δ	1 : 128

Preparation of small intestine.

Lengths of small intestine were obtained from rabbits killed by a blow on the neck. When not in use the gut was stored in Ringer solution at 4°. For the test, 4 cm. open segments were suspended in 60 ml. of oxygenated Ringer at 38°. Toxic filtrates were warmed to 38° and added to the bath after the removal of an equivalent volume of Ringer solution. All recordings were made on smoked drums rotating at 1 mm. per second.

RESULTS.

Examination of filtrates derived from pyogenic strains showed that amounts varying between 1 ml. (1·6 per cent) and 5 ml. (8·3 per cent) could be added to the bath without significant alteration in tone or amplitude of contraction. The control filtrate from uninoculated media occasionally caused a loss of tone as previously noted by Richmond *et al.* (1942). Even those filtrates with a high α-haemolysin titre failed to produce a response. In view of Fulton's observations on the effect of intraperitoneal β-lysin (Fulton, 1943), it was of interest that filtrates from the two β-lysin-producing strains (R.K. and Hillman) were also negative under the conditions of the test (Fig. 1).

Filtrates from the twelve food-poisoning strains were tested under identical conditions. The addition of 1 ml. amounts of toxin produced a slight and transient increase in tone (Fig. 2). The addition of successively larger quantities to the same segment of gut resulted in the response seen in Fig. 3. It was eventually found that approximately 4 ml. (6·6 per cent) of filtrate would produce a well-defined response and this quantity was used in all subsequent experiments. During examination of the twelve filtrates, three variations in response were observed which closely resembled those described by Richmond *et al.* (1942). In all experiments there was increased tonicity. One preparation reacted with almost total spasm (Fig. 4), in another there was an increased amplitude of contraction (Fig. 5), while the remainder showed no alteration in amplitude.

This property of the food-poisoning filtrates was remarkably stable to heat, being unaffected by heating to 56° for ½ hr. (Fig. 6) or by boiling and cooling rapidly. This agrees with the observations of Davidson and Dack (1939) and Surgalla and Hite (1945). The addition of sufficient staphylococcus antitoxin to neutralise α-haemolytic activity also failed to abolish the effect (Fig. 7). The increased tonicity could be reversed by atropine (Fig. 8). A single experiment was performed using kittens' small intestine which responded in a similar manner, but was no more sensitive than rabbit preparations. On account of the large volume of filtrate required to elicit a response it was felt that the media and conditions of culture, although optimal for haemolysin production, might not be ideal for the production of enterotoxin. Consequently, several food-poisoning strains were grown in Robertson's cooked meat medium at 30° for 18 hr. in an attempt to reproduce the conditions under which clinical outbreaks have been known to occur. The filtrates from these cultures showed no α-haemolytic activity and quantities up to 10 ml. failed to produce any response from isolated rabbit small intestine.

DISCUSSION.

The dicrepancy between the reults obtained by Bayliss (1940) and Richmond *et al.* (1942) can in part be explained by the volume of toxic filtrate employed in the tests. Bayliss failed to elicit a response with between 0·1 and 1·0 per cent toxin in the bath, while Richmond *et al.* (1942) used 10 ml. (16·6 per cent) to obtain an effect. Although the latter quantity is larger than that used in the present experiments (6·6 per cent), the diminished potency of Richmond's toxins may well be accounted for by filtration before use. Since Seitz filtration caused a 30-fold reduction in the α-haemolysin titre of filtrates, it was felt that other toxic constituents might well be adsorbed during sterilization and this was

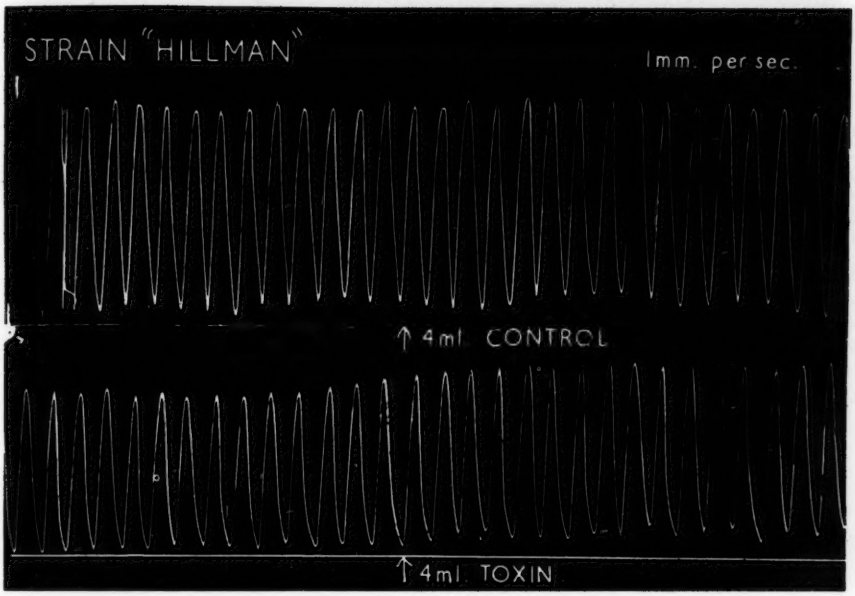


FIG. I.—Lack of response on addition of 4 ml. of toxic filtrate containing β-lysin from pyogenic strain.

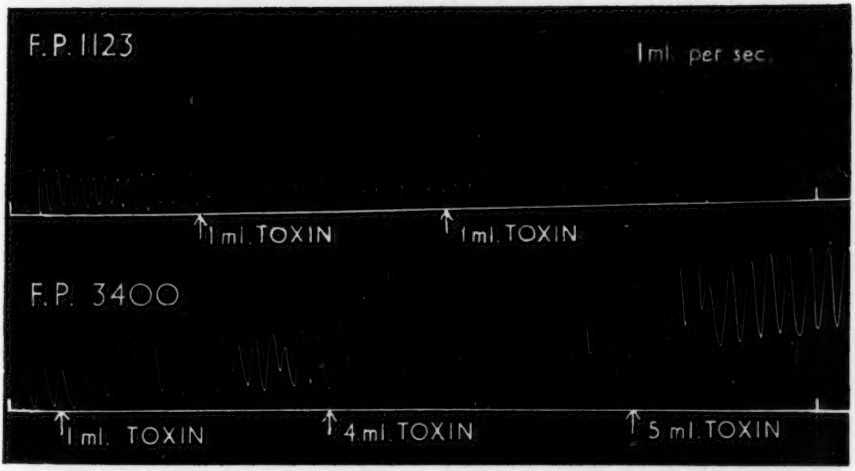


FIG. 2 and 3.—Increased tonicity obtained with varying quantities of filtrate from food-poisoning strain. (× ½.)

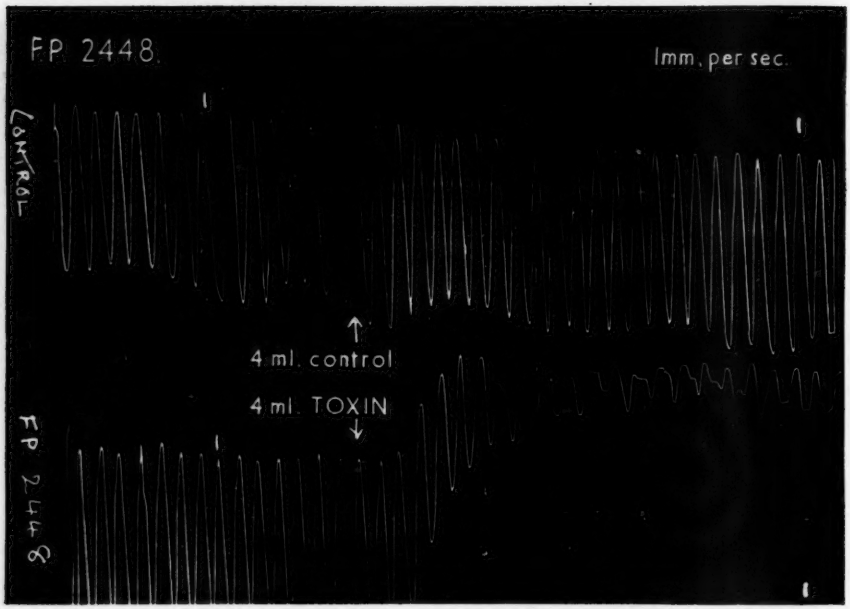


FIG. 4.—Increase in tonus with almost total spasm after addition of toxic filtrate.

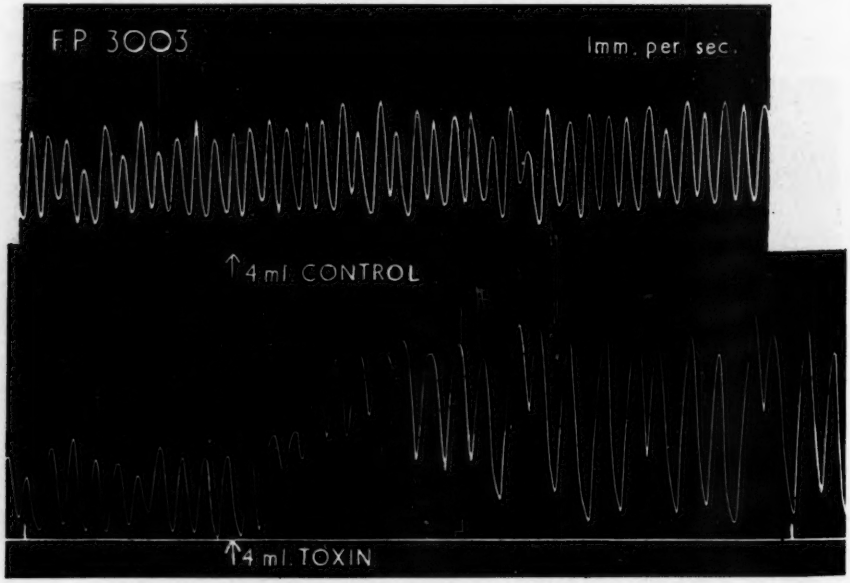


FIG. 5.—Increased amplitude of contraction with increased tonus: 4 ml. of filtrate from food-poisoning strain.

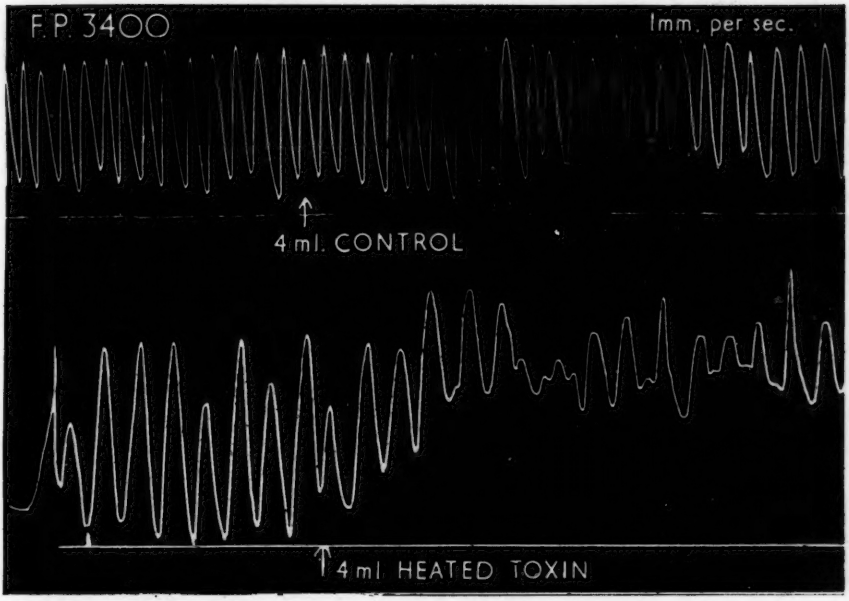


FIG. 6.—Stability of filtrate to heating to 56° for ½ hr.

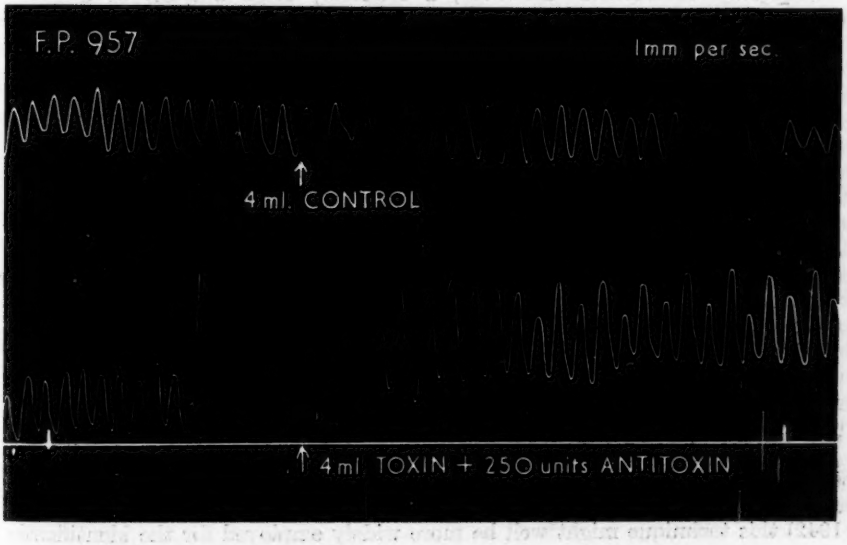


FIG. 7.—Failure of staphylococcus antitoxin to neutralise enterotoxin.

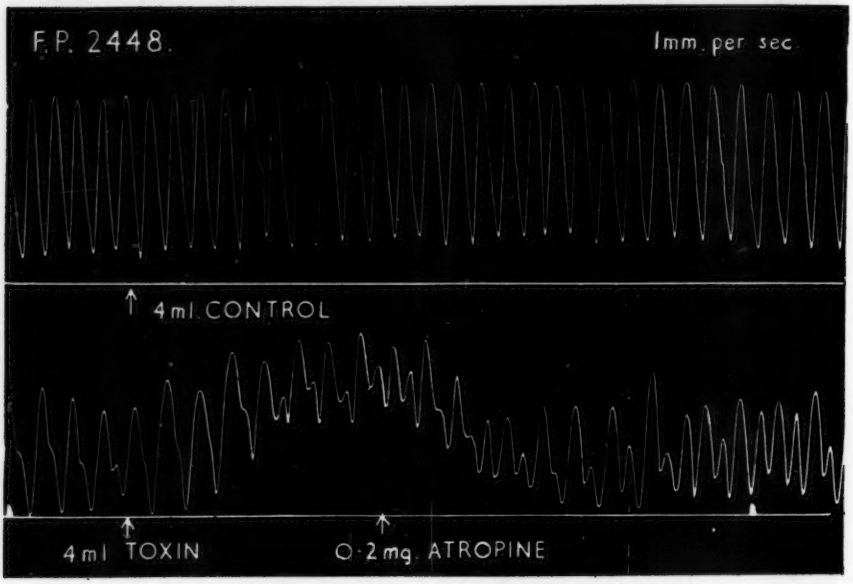


FIG. 8.—Reversal of increased tonus by the addition of atropine.

consequently abandoned. However, a relatively large amount of unfiltered toxin was still required to produce a response, doubtless owing to the insusceptibility of the rabbit, for the human volunteer clearly remains the most sensitive test of enterotoxin production. None of the food-poisoning strains tested produced β-lysin, but observations on the two pyogenic strains elaborating this toxin show that β-lysin does not elicit a response and that its production by an enterotoxic strain would not alter the validity of the test. This, together with the fact that α-lysin in high titre fails to elicit a response, confirms the observation of Surgalla and Hite (1945) that the haemolysins and the enterotoxin are separable and distinct entities. The conclusions to be drawn from the experiments of Richmond *et al.* (1942) are limited by the fact that only one enterotoxic strain seems to have been used for toxin production in 54 trials under varying conditions. Furthermore, the control organism was an air-borne strain of staphylococcus. The present series of experiments indicates that the ability to affect rabbit small intestine is a property possessed by several food-poisoning staphylococci, and is not confined to one particular strain. Pathogenic staphylococci isolated from human lesions do not appear to possess this property and may therefore be distinguished from those pathogens which also produce enterotoxin.

It would appear, therefore, that food-poisoning staphylococci give rise to toxic filtrates capable of producing a characteristic response when added to isolated rabbit small intestine. As originally suggested by Richmond *et al.* (1942) this technique might well be more widely employed for the identification of enterotoxic strains.

SUMMARY.

Twenty-four strains of *Staph. pyogenes* were examined for haemolysin production. Toxic filtrates were prepared and tested on isolated rabbit small intestine.

All twelve food-poisoning strains produced a characteristic increase in tone with not less than 4 ml. of filtrate. No response was obtained with filtrates from twelve pyogenic strains.

This property was remarkably thermostable. It was not neutralized by staphylococcus antitoxin, but was reversed by atropine. It was not related to the production of staphylococcal haemolysins. It is suggested that the method be more widely explored for the recognition of enterotoxin-producing strains of *Staph. pyogenes*.

I am indebted to Professor G. Payling Wright and Dr. J. Howard for their valuable advice, and also to Dr. R. E. O. Williams for supplying the twelve food-poisoning strains and for his interest in this problem.

I also wish to thank the Department of Medical Illustration, Guy's Hospital, for reproduction of the tracings and the Department of Pharmacology, Guy's Hospital, for loan of equipment.

REFERENCES.

Barber, M. A.—(1914) *Philipp. J. Sci.* (*b*), **9**, 515.
Baylis, M.—(1940) *J. exp. Med.*, **72**, 669.
Borthwick, G. R.—(1933) *Brit. J. exp. Path.*, **14**, 236.
Dack, G. M., Cary, W. E., Woolpert, O., and Wiggers, H.—(1930) *J. prev. Med., Baltimore*, **4**, 167.
Davison, E., and Dack, G. M.—(1939) *J. infect. Dis.*, **64**, 302.
Denys, J.—(1894) *Bull. Acad. Med. Belg.*, **8**, 496.
Dolman, C. E., Wilson, R. J., and Cockcroft, W. H.—(1936) *Can. pub. Hlth. J.*, **27**, 489–493.
Iidem—(1938) *J. Immunol.*, **35**, 13.
Elek, S. D., and Levy, E.—(1950) *J. Path. Bact.*, **62**, 541.
Fulton, F.—(1943) *Brit. J. exp. Path.*, **24**, 65.
Jordan, E. O., and McBroom, J.—(1931) *Proc. Soc. exp. Biol., N.Y.*, **29**, 161.
Owen, R. W. G.—(1907) *Phys. and Surg., Ann. Abor.*, **29**, 289.
Richmond, J. J., Reed, C. I., Shaughnessy, H. J., and Michael, V.—(1942) *J. Bact.* **44**, 201.
Stone, R. V.—(1935) *Proc. Soc. exp. Biol., N.Y.*, **33**, 189.
Surgalla, M. J., and Hite, K. E.—(1945) *J. infect. Dis.*, **76**, 78.
Van Heyningen, W. E.—(1950) " Bacterial Toxins." Oxford (Blackwell Scientific Publications), p. 73.

A STUDY OF THE TREPONEMA IMMOBILIZING ANTIBODY IN SYPHILIS.

C. W. CHACKO.

From the Wright-Fleming Institute of Microbiology, St. Mary's Hospital Medical School, London, W.2.

Received for publication June 3, 1953.

WHEN man or animal is infected with *Treponema pallidum*, it is probable that at least two different "antibodies" are produced. The Wasserman antibody or "reagin" is one of them and it forms the basis of the standard serological tests for syphilis in current use. Reagin is considered to be antilipoidal in nature because the lipoidal antigen of the standard tests reacts with it, fixing the complement or resulting in flocculation. However, the standard tests are sometimes positive in diseases other than syphilis and even in apparently healthy persons, so that reagin must be regarded as a relatively non-specific antibody. The nature of its origin in syphilis is still a matter of controversy.

The presence of another more specific antitreponemal antibody in syphilis was first reported by Eberson (1921) who demonstrated its presence in syphilis by protection tests in rabbits. Turner (1939) and Turner, Kluth, McLeod and Windsor (1948) confirmed Eberson's findings in more extensive *in vivo* and *in vitro* protection tests in rabbits. Then, Nelson and Mayer (1949), using the virulent Nichols strain of *Treponema pallidum* grown in and extracted from rabbit testes and kept alive in a special anaerobic medium, showed that the actively motile treponemes were immobilized when incubated in the presence of syphilitic serum and fresh complement. On the basis of this immobilizing antibody in syphilitic sera, Nelson and Mayer devised their Treponema Immobilization Test (T.P.I.) for syphilis. Studies with sera and cerebrospinal fluid from cases of syphilis and from various other infections and diseases have shown that this immobilizing antibody, in contrast with reagin, is specific for treponemal infections, particularly syphilis (Nelson and Mayer, 1949; Nelson, Zeutlin, Dissendruck and Austin, 1950; Nelson, Zeutlin, Dissendruck, Stack and Barnet, 1951; Magnuson and Thomson, 1949; Mohr, Moore, and Hill, 1950; Durel, Sousse and Borel, 1952).

The purpose of the present work has been to study experimentally the relationships of reagin and the treponemal antibody in syphilitic human and rabbit sera, with the use of the T.P.I. test and standard tests. The stability of the treponemal antibody under varying conditions has also been examined.

MATERIALS AND METHODS.

Live treponemal suspension.

The Nichols strain of *T. pallidum* obtained from the School of Hygiene, Johns Hopkins University, was used, and was maintained in serial culture in the testes of young adult rabbits of mixed breed, free from rabbit spirochaetosis and kept in individual cages. It produced a diffuse orchitis in rabbit testes in 7 to 10 days, when about 0·5 ml. of a testes suspension containing 15–20 million *T. pallidum* per ml. was inoculated into each testis. The rabbit testes, after inoculation, were palpated daily from the fifth day onwards. At the earliest

signs of syphilitic orchitis (diffuse induration and enlargement confirmed by dark field microscopic examination of aspirated fluid), the testes were removed aseptically, cut into thin slices and ground into a suspension, using a sterile mortar and pestle with a little sterile fine sand and 5 ml. per testis of sterile 50 per cent normal rabbit serum in normal saline. The suspension was centrifuged lightly, the supernatant collected and its content of *T. pallidum* was checked under dark field microscopy: 15–20 *T. pallidum* per field corresponded to the strength required to produce orchitis in 7–10 days.

The infected rabbit was examined at least twice a week for orchitis which usually developed after about 7–10 days. Within the next 48 hours the rabbit was killed by injecting air intravenously. The scrotum was clamped and cut off, and in a glass operating chamber, was cleaned with absolute alcohol and incised: the testes were removed aseptically, and after discarding the cord, epididymis and fat, were transferred to a sterile petri dish. Each testis was then cut once longitudinally and then transversely into about 10 thin slices with a sterile sharp razor blade in a special holder. The pieces were washed with cold sterile normal saline to remove any damaged tissue cells and transferred to about 20–30 ml. of Nelson's medium contained in a 100 ml. sterile extraction flask, closed by a ground glass stopper with a stopcock. The flask was filled with a mixture of 95 per cent N_2 and 5 per cent CO_2 by three successive evacuation and refilling operations with a special evacuation apparatus. The flask and the contents were then gently agitated by rocking in a special water bath/rocking apparatus at 35°. The treponemes were extracted into the medium alive and motile, and rocking was continued until about 5–8 treponemes per high dry field of the dark field microscope were obtained. Usually, at the end of an hour of rocking, the concentration of treponemes in the suspension was checked under the microscope and rocking was continued if necessary to obtain the desired number per field. The extract was now transferred to sterile 10 ml. centrifuge tubes and spun at 1,500 r.p.m. for 7 minutes to get rid of tissue debris, red cells and spermatozoa. The tissue-free suspension of *T. pallidum* forms the antigen in the T.P.I. test. The rest of the testis tissue can be used as a suspension to infect a new rabbit.

The medium for preserving the viability of the treponemata under anaerobic conditions was that described by Nelson and Mayer (1949). As some of the ingredients are not easily available the following notes may be helpful.

The bovine plasma fraction V was obtained from Armour and Co., Ltd.; ultra filtrate of bovine serum (Sims) was obtained from Microbiological Associates Inc., Coral Gables, Florida, U.S.A. and was stored under an atmosphere of 5 per cent CO_2 at 4°. Sodium thioglycollate and sodium pyruvate were obtained from George T. Gurr Ltd. and were stored dry in crystalline form in a desiccator under vacuum at 4°; glutathione from Roche Products Ltd. and L-cysteine hydrochloride from Ash Laboritories Ltd. were stored at 4°. The stock solutions of the individual constituents of the medium were stored at −20°. Immediately before use the stored solutions were rapidly thawed, the medium was made up according to the proportions given in the formula, and was sterilized by filtration through a gradocol membrane filter (0·7μ diameter) under slight negative pressure.

The water bath extraction apparatus was made to order by Charles Hearson and Co. Ltd. It was 24 in. × 12 in. × 18 in. (60 × 30 × 45 cm.) and was electrically controlled at 30–40° range, ordinarily working at 35°. The extraction flask, clipped to a horizontal metal bar fixed on the top of the bath was immersed in the water and swung backwards and forwards at speeds varying from 10–60 per minute by a small electrically driven motor and resistance. The flask was 100 ml. in capacity, flat-bottomed, made of pyrex glass and had a ground glass stopper with a stopcock which was lubricated with Dow Corning Silicone high vacuum lubricant.

A Zeiss binocular microscope was used with 10× eye piece and $\frac{1}{8}$ in. objective (dry) and cardioid dark ground condenser, and was illuminated by a McCartney microscope lamp (R. R. Beard Ltd.) fitted with a heat-absorbing filter.

Rimless pyrex test tubes 13 × 100 mm. were used, capped with Oxoid (Oxo Ltd.) aluminium caps of half inch diameter.

All the glassware was cleaned with chromic acid solution, thoroughly rinsed in tap water and then distilled water and sterilized before use.

Serum Specimens.

The syphilitic and non-syphilitic sera of human origin were obtained from the Special (V.D.) Clinic and other departments of St. Mary's Hospital. The syphilitic rabbit sera were

obtained from rabbits experimentally infected intratesticularly in the laboratory and left untreated for 8–12 months. All sera were preserved at −20° until ready for testing. Pooled guinea-pig serum preserved at −20° in 5 ml. sterile screw-capped bottles was used as the source of complement in the tests. About 80–100 M.H.D. of complement per unit volume (0·1 ml.) was usually obtained by titration to the 100 per cent haemolysis end point, using 3 per cent sensitized sheep cells in a total volume of 0·4 ml.

T.P.I. test technique.

The technique used was essentially the same as that used by Nelson and Mayer (1949) and Nelson *et al.* (1951). The titre of positive sera was expressed as the reciprocal of the serum dilution which produced immobilization of 50 per cent of the treponemes in the presence of a 1 : 10 dilution of complement after 18 hours' incubation at 35°., *i.e.*, the $I.T._{50}$ (18 hr.). Titres given in the tables were obtained by plotting the percentage immobilization against the reciprocal of the serum dilution on semi-logarithmic graph paper and the 50 per cent end point was determined by linear interpolation.

The Venereal Diseases Research Laboratory (V.D.R.L.) slide flocculation test with cardiolipin antigen (Harris, Rosenburg and Reidal, 1946; Harris, Rosenburg and Delveechio, 1948) was used as the standard test. The titre was expressed as the reciprocal of the greatest serum dilution that produced a positive reaction.

EXPERIMENTS AND RESULTS.

The Relationship of the Treponema Immobilizing Antibody and Reagin in Syphilitic Sera.

The relationship of the treponema immobilizing antibody to reagin was studied in experimental rabbit syphilis. Five young adult male rabbits which were initially sero-negative with the T.P.I. test and the V.D.R.L. slide flocculation test, were inoculated intratesticularly with the Nichols strain of *T. pallidum*. The infection was allowed to proceed untreated for 8–12 months. About 10 ml. blood was withdrawn aseptically from the heart under anaesthesia every month after infection. The separated sera were stored at −20° until used. Eight to twelve specimens of serum were obtained from each rabbit. Doubling dilutions of the serum from each rabbit were made in 0·85 per cent sterile saline and quantitative tests were made with the T.P.I. test and V.D.R.L. slide flocculation tests in parallel. The titres of the two antibodies obtained in 5 rabbits are tabulated in Table I. It will be noted that the reagin antibody in syphilitic rabbits reaches its maximum titre during the first or second month of infection then falls to near to normal level within the next few months whereas the immobilizing antibody titre rises more slowly to its maximum but continues at a high level for 8–12 months.

This apparently diverse nature of the two antibodies was investigated by absorption experiments. The V.D.R.L. cardiolipin antigen was diluted 1 : 10 with buffered saline solution and two 5 ml. quantities were centrifuged at 10,000 r.p.m. for 30 minutes in a cold centrifuge at 5°. The supernatants were discarded and 5 ml. pooled human syphilitic sera was added to the deposit of antigen particles in one tube. The mixture was agitated thoroughly for 15 minutes. The resulting flocculate was separated from the serum by centrifugation in the cold centrifuge at 10,000 r.p.m. for 30 minutes; 2·5 ml. of the supernatant serum was set apart marked "absorbed serum I." The other 2·5 ml. was then added to the antigen deposit in the other centrifuge tube, agitated and a second absorption and centrifugation was carried out as before. This latter serum was marked "absorbed serum 2." The original serum and the two absorbed sera were tested

TABLE I.—*Comparative Titres of T.P.I. Antibody and Reagin in Untreated Experimental Rabbit Syphilis.*

Rabbit.	Tests.	Months. 1st.	2nd.	3rd.	4th.	5th.	6th.	7th.	8th.	9th.	10th.	11th.	12th.
JH 1	T.P.I.	40	100	80	85	160	190	nt	300	320	305	640	360
	V.D.R.L. cardiolipin	4	32	8	4	4	2	nt	2	+	+	+	±
JH 5	T.P.I.	25	60	60	125	180	180	200	nt	320	180	320	nt
	V.D.R.L. cardiolipin	32	4	+	±	±	±	±	nt	±	±	±	nt
JH 7	T.P.I.	±	160	170	190	160	270	320	nt	nt	nt	nt	nt
	V.D.R.L. cardiolipin	32	4	+	±	±	±	−	−	nt	nt	nt	nt
JH 10	T.P.I.	75	190	nt	nt	350	300	520	460	nt	nt	nt	nt
	V.D.R.L. cardiolipin	64	16	nt	nt	8	±	±	±	−	−	nt	nt
JH 20	T.P.I.	−	nt	20	80	160	110	40	110	nt	nt	nt	nt
	V.D.R.L. cardiolipin	−	nt	32	16	4	2	±	±	nt	nt	nt	nt

nt = not tested, + = positive, ± = weak positive, and − = negative in undiluted serum.
The sera of all rabbits were negative before infection.

in parallel with the T.P.I. test and the V.D.R.L. slide flocculation test quantitatively. This absorption experiment was repeated with two rabbit syphilitic sera and the results obtained are given in Table II.

The reagin appears to have been removed by the cardiolipin antigen, leaving the immobilizing antibody little affected.

TABLE II.—*Result of Absorption of the Reagin from Syphilitic Sera with Cardiolipin Antigen and Quantitative Testing of their Immobilizing Antibody Content.*

Tests.	Serum A. (Human syphilitic pool.) Unabsorbed.	Ab-sorbed once.	Ab-sorbed twice.	Serum B. (Syphilitic rabbit serum.) Unabsorbed.	Ab-sorbed once.	Ab-sorbed twice.	Serum C. (Rabbit syphilitic pool.) Unabsorbed.	Ab-sorbed once.	Ab-sorbed twice.
T.P.I. titre	90	110	100	50	60	50	180	165	170
V.D.R.L. titre	4	−	−	8	−	−	16	±	−

The figures represent the reciprocals of the highest dilution of serum giving a positive result.
− = Negative.

Stability of the Immobilizing Antibody.

In freeze-dried syphilitic sera.—A pool of sterile human syphilitic serum was dried from the frozen state in 0·2 ml. quantities, using a centrifugal freeze-drier (Edwards & Co.); 24 tubes were prepared, sealed in vacuum and stored at room temperature. At monthly intervals these freeze-dried sera were re-constituted with sterile distilled water and tested quantitatively in duplicate with the T.P.I. test and compared in parallel with the original serum preserved at −20°. The results showed that there was no significant change in the titre of sera stored in the dry state for 4 months.

In syphilitic sera under varying temperatures.—A pool of syphilitic rabbit sera was heated in duplicate at 56° for 1 hour and 2 hours in 3 ml. quantities. The

sera were then tested quantitatively in duplicate with the T.P.I. test and the titres compared with that of the original unheated serum. The results indicated that the immobilizing antibody was not affected by this degree of heating.

Two syphilitic sera and a syphilitic cerebrospinal fluid were left in duplicate in 5 ml. screw-capped bottles on the bench at room temperature (16–23°). At weekly intervals they were tested quantitatively along with their originals, which had been preserved at −20°. Results showed that the immobilizing antibody was unaffected by 2–4 weeks' storage at room temperature.

In contaminated syphilitic sera.—Three ml. of a pool of syphilitic human sera was experimentally inoculated in duplicate with *Staphylococcus aureus* and left in the incubator at 37° for 48 hours. Another 3 ml. of the same pooled sera was left on the bench in duplicate exposed to laboratory contaminants at room temperature for 10 days. At the end of these periods, when they were shown to be grossly contaminated, the sera were filtered free of bacteria by passage through gradocol membranes and tested quantitatively with the T.P.I. test, comparing the titres obtained with those of the original sera which had been preserved at −20°. The results indicated that the T.P.I. antibody withstands these unfavourable conditions remarkably well.

In vivo *Sensitization of* T. pallidum *by its own Antibody.*

A rabbit was infected intratesticularly by the technique already described to produce an orchitis in 7–8 days. At the earliest signs of orchitis, confirmed by dark field observation of aspirated fluid the right testis was removed aseptically under anaesthesia. With a suspension of *T. pallidum* extracted from this testis, a T.P.I. test was performed on known negative and known positive rabbit sera, serially diluted beyond their probable titres and the percentage immobilized by each dilution was noted. Four days later the rabbit was sacrificed and the left testis was used to prepare a suspension of live treponemes. A T.P.I. test was performed using this suspension with similar serial dilutions of the sera preserved at −20°, and the percentage of immobilized treponemes was compared with those obtained with the right testis treponemes.

This experiment was repeated with another rabbit from which the two testes were removed at intervals of 7 days and the extracted treponemes tested against 4 normal human sera qualitatively and one positive rabbit serum quantitatively.

The results (Tables III and IV) show that the T.P.I. test done with suspension prepared from rabbit testes harvested 4 days or more after orchitis is detectable, gives a false positive test on normal sera and an increase of the titre of positive sera far beyond a chance variation of its previous known titre.

DISCUSSION.

The treponema immobilization test has made available an *in vitro* technique for studying the relation of the specific antitreponemal antibody to reagin. The quantitative study of their titres in untreated rabbit syphilis indicates that the immobilizing and reagin activities are due to different antibodies. Although the titres of the two antibodies tested monthly are not strictly comparable, their different courses—the immobilizing antibody maintaining a steady level after reaching the peak while the reagin disappears again—strongly suggests their

distinct nature. A similar study in untreated human syphilis would be illuminating but the practical difficulties in allowing human syphilis to progress untreated for a sufficient length of time would be great. It has however been shown by other workers who examined sera from various stages of human syphilis from different individuals, that there is no correlation between the results of the two types of tests except perhaps in early syphilis. Again, in non-treponemal infec-

TABLE III.—*Effect on T.P.I. Test of using Treponema Suspension from Rabbit Testes beyond the stage of Early Orchitis.*

Sera.	Dilutions.	Treponemes immobilized per cent.	
		Suspension from testis on 2nd day of orchitis.	Suspension from testis on 4th day of orchitis.
Negative rabbit serum :			
J.3	1/10	17	100
,,	1/20	9	100
,,	1/40	4	92
,,	1/80	0	83
,,	1/160	0	73
,,	1/320	0	67
,,	1/640	0	62
Positive rabbit serum :			
J.19	1/10	100	100
,,	1/20	59	100
,,	1/40	31	95
,,	1/80	0	91
,,	1/160	0	86
,,	1/320	0	59
,,	1/640	0	55
Serum-free saline control		2	2

TABLE IV.—*Effect of using Treponema Suspension Extracted from Rabbit Testes beyond the Early Stages of Orchitis on T.P.I. Tests Results.*

Sera.	Dilution.	Treponemes immobilized per cent.	
		Suspension from testis on 1st day of orchitis.	Suspension from testis on 7th day of orchitis.
Negative human serum :			
1	1/10	0	55
2	1/10	17	68
3	1/10	11	55
4	1/10	10	71
Positive rabbit serum :			
J56	1/10	100	100
,,	1/20	84	100
,,	1/40	75	100
,,	1/80	50	100
,,	1/160	30	85
,,	1/320	0	83
,,	1/640	0	71
Serum-free saline control		10	5

tions and diseases, the immobilizing antibody is always absent while reagin may be present. In late syphilis, particularly in latent syphilis, when resistance to reinfection is usually high, the level of the immobilizing reagin is often low (Magnuson and Thomson, 1949 ; Nelson *et al.*, 1950 ; Chacko, 1953).

This lack of relationship was further shown in absorption experiments when reagin could be removed from syphilitic sera without affecting the treponemal antibody. Attempts made to cross-absorb one or both antibodies from syphilitic sera with live and killed virulent *T. pallidum* were unsuccessful because of the difficulties in obtaining an adequate strength of tissue-free *T. pallidum* for absorption. The importance of cross-absorption experiments is stressed, as they may also indicate the presence of lipoidal and other antigenic constituents in virulent *T. pallidum* which is concerned with the production of multiple antibodies of specific and non-specific nature.

The stability of treponema immobilizing antibody to heat, freeze-drying, and contamination has been investigated. It is moderately heat-stable, and can be preserved for months unaltered by freeze-drying. Infected sera can be sterilized by filtration without appreciable loss of antibody titre. The use of a preservative like merthiolate is contra-indicated in the T.P.I. test owing to its treponemocidal nature. However, contaminated serum may be filtered free of bacteria without damaging its immobilizing antibody content significantly.

Immobilizing antibody will also remain unchanged when dried from the frozen state and stored at room temperature at least for 4 months. The practical application of this characteristic of the antibody would be in the preservation of standard positive syphilitic sera for control and reference purposes.

Nelson *et al.* (1951) described the phenomenon of sensitization of *T. pallidum* by immobilizing antibody produced in rabbit syphilis when the orchitis was allowed to proceed beyond its earliest stages. When these treponemes came in contact with complement *in vitro*, they were immobilized. Thus the treponemes harvested from orchitis beyond a certain stage and used as antigen, interfered with the specificity of the T.P.I. test by producing false positive tests for syphilis in normal and non-syphilitic sera. This finding was confirmed in the present study. It is difficult to say what the significance of this phenomenon may be in the natural infection in man.

SUMMARY.

The Treponema Pallidum Immobilization (T.P.I.) test has been applied to the study of the antibodies in syphilis.

The titres of the immobilizing antibody and the Wasserman antibody or reagin were followed in untreated experimental rabbit syphilis by parallel quantitative tests of sera collected at monthly intervals, and were found to follow different courses showing little correlation with each other. The distinct nature of the two antibodies has been further demonstrated by absorption of the reagin alone from syphilitic sera with cardiolipin antigen.

The immobilizing antibody, which is specific in treponemal diseases, particularly in syphilis, is remarkably stable in syphilitic sera when preserved under varying conditions of temperature and in contaminated sera. These characteristics of the antibody have been considered of practical importance in the use of the T.P.I. test as a specific reference test for syphilis.

The occurrence of a phenomenon of *in vivo* sensitization of *T. pallidum* by its own immobilizing antibody in rabbit testes has been confirmed. The significance of this phenomenon is that it would cause false positive tests for syphilis with the T.P.I. test.

I acknowledge my gratitude to Sir Alexander Fleming for laboratory facilities at the Wright-Fleming Institute of Microbiology ; Professor Robert Cruickshank and Dr. R. H. Gorrill for guidance and useful advice ; Professor T. B. Turner and Dr. Robert Nelson of Johns Hopkins University School of Hygiene, U.S.A. for supplying the Nichols virulent strain of *T. pallidum* and technical advice on the performance of the T.P.I. test ; and the Government of Madras, India for study leave abroad.

REFERENCES.

Chacko, C. W.—(1953) *J. clin. Path.*, **6**, 227.
Durel, P., Sousse, A., and Borel, L.—(1952) *Brit. J. ven. Dis.*, **28**, 68.
Eberson, F.—(1921) *Arch. Derm. Syph.*, **4**, 49.
Harris, A., Rosenberg, A. A., and Reidal, L. M.—(1946) *J. Vener. dis. Inform.*, **27**, 169.
Iidem and Delveechio, E. R.—(1948) *Ibid.*, **29**, 72.
Magnuson, H. J., and Thomson, F. A.—(1949) *Ibid.*, **30**, 309.
Mohr, C. F., Moore, J. E., and Hill, J. H.—(1950) *Amer. J. Syph.*, **34**, 405.
Nelson, R. A., Jr., and Mayer, M. M.—(1949) *J. exper. Med.*, **89**, 369.
Idem, Zeutlin, H. E. C., Dissendruck, J. A., and Austin, P. G. N.—(1950) *Amer. J. Syph.*, **34**, 101.
Iidem, Stack, P. S., and Barnet, M.—(1951) *J. Immunol.*, **66**, 667.
Turner, T. B.—(1939) *J. exp. Med.*, **69**, 867.
Idem, Kluth, F. C., McLeod, C., and Windsor, C. P.—(1948) *Amer. J. Hyg.*, **48**, 173.

THE HAEMOLYSIN OF *CLOSTRIDIUM HISTOLYTICUM*.

J. G. HOWARD.*

From the Central Public Health Laboratory, Colindale, London, N.W. 9.

Received for publication June 4, 1953.

ALTHOUGH the production of a haemolysin by *Cl. histolyticum* is well established (Mita, 1934), its properties have been little investigated. It is not formed in a typical peptone infusion broth, but Stewart (1936) found that the addition of 2 per cent glucose to media of this type initiated haemolysin formation. This was attributed to the reducing property of the sugar. More recently Bowen (1952) discovered that this haemolytic toxin was oxygen-labile, being inactivated within a few days by storage in the cold in either air or oxygen. Haemolytic activity was restored by the addition of a reducing agent. A much slower and permanent loss of haemolysin occurred during storage, even under reduced conditions.

A well-defined group of bacterial haemolysins (streptolysin O, tetanolysin, pneumolysin and *Cl. welchii* θ toxin) all possess common properties. They are reversibly inactivated by oxidising agents, inhibited by cholesterol and show an immunological inter-relationship (van Heyningen, 1950). This latter property was investigated by Todd (1934, 1941), who demonstrated that an oxygen-labile haemolysin is neutralised by the antibody prepared against any one of the others of the group, but not by the antibody of an oxygen-stable haemolysin (*e.g.*, staphylococcus α toxin). Another significant group feature is the resistance exhibited by mouse red cells to these haemolysins. This varies between a moderate resistance to tetanolysin and a remarkably high resistance to streptolysin O. (Howard and Wallace, 1953).

The present investigation was undertaken to determine whether *Cl. histolyticum* haemolysin shared these properties, in addition to that of oxygen-lability, with the other members of this group of bacterial toxins.

MATERIALS AND METHODS.

The Weinberg strain of *Cl. histolyticum* was used (National Collection of Type Cultures No. 503). One litre of an ordinary peptone infusion broth, with the addition of 2 per cent glucose and 0·02 per cent sodium thioglycollate, was distributed into four flasks. Each was inoculated with 2·5 ml. of a 24 hr. broth culture of *Cl. histolyticum* and incubated anaerobi-

* Present address, The David Bruce Laboratories, East Everleigh, nr. Marlborough.

DESCRIPTION OF PLATE.

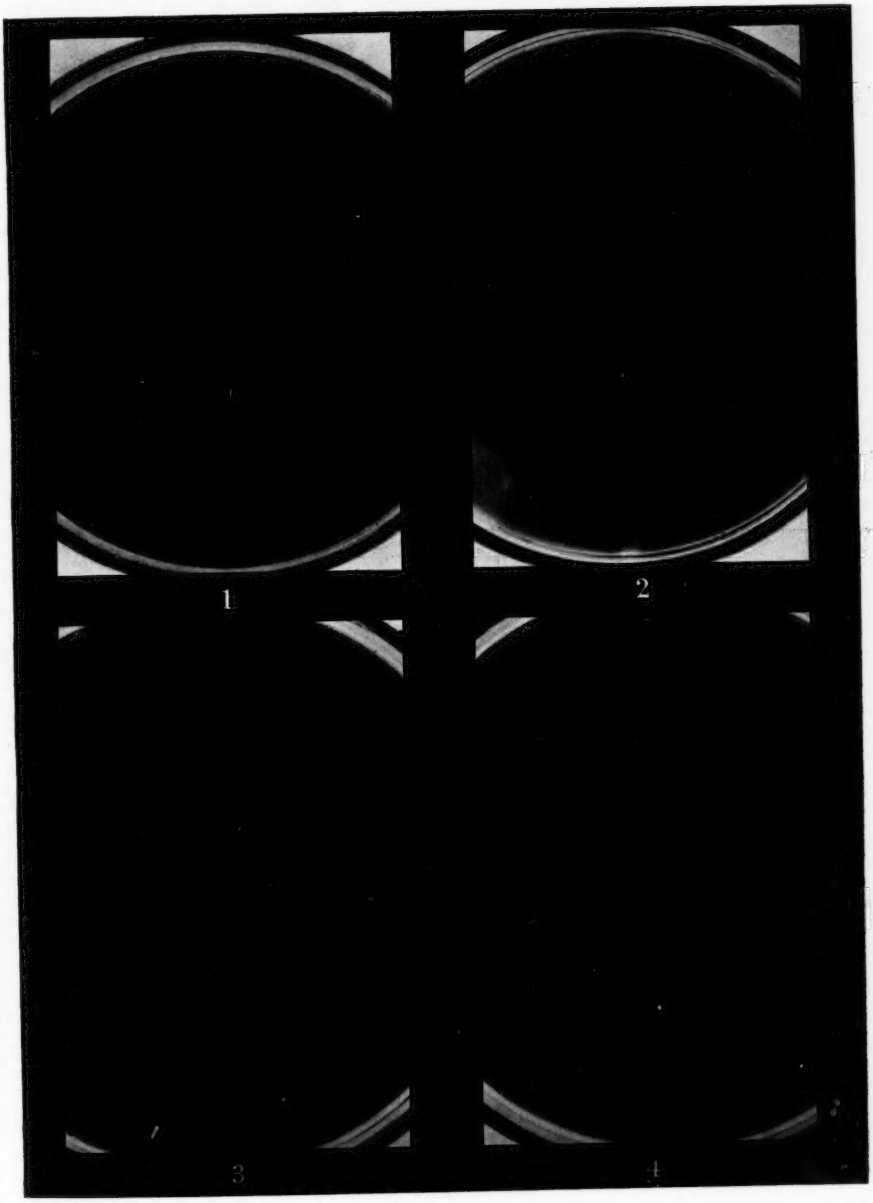
The inhibition of four oxygen-labile haemolysins by antistreptolysin O.

FIG. 1.—*Cl. histolyticum*. The streak of growth is not shown. The underlying zone of haemolysis is clearly inhibited.

FIG. 2.—*Pneumococcus*. The streak of growth is clearly seen. The surrounding area of haemolysis is sharply inhibited.

FIG. 3.—*Cl. welchii*, type A. Adjacent to the streak of growth is a narrow zone of complete haemolysis, due to θ toxin, inhibited by antistreptolysin O. The larger outer zone of partial haemolysis, due to α toxin is uninhibited.

FIG. 4.—*Cl. tetani*. The growth itself is not shown. The underlying haemolysis, completely inhibited by antistreptolysin O, traces the spreading colonies.



Howard.

cally at 37° for 18 hr. The cultures were centrifuged at 3000 r.p.m. for 30 minutes. The supernatants were then withdrawn for titration of haemolysin.

Titrations.—Serial doubling dilutions of culture supernatant were made with normal saline in conical centrifuge tubes. To 2·0 ml. of each dilution was added 0·5 ml. of 0·2 M sodium thioglycollate. The tubes were placed in a 37° water bath for 10 min. to allow full reduction to take place. An equal volume (2·5 ml.) of a 2·5 per cent suspension of rabbit red cells in saline was added to each tube. After a further 30 min. incubation, the tubes were centrifuged and the percentage haemolysis estimated after the method described by Herbert (1941).

Effect of oxidation.—In place of the thioglycollate, 0·5 ml. of 0·2 M hydrogen peroxide was added to each dilution. Titrations were carried out in the manner described.

Effect of exposure to cholesterol.—Activated haemolysin was exposed to cholesterol at a ligroin-water interface, using the method described by Howard *et. al.* (1953). Undiluted lysin (5 ml.) was reduced by the addition of 1 ml. of 0·2 M thioglycollate, and 50 ml. of a 0·1 per cent solution of cholesterol in ligroin (B.D.H.) perfused through it. The effect of the perfusion of 50 ml. of ligroin alone through haemolysin was tested as a control. The haemolytic activity of a control solution of activated lysin which had stood for a similar period at room temperature was also estimated.

Comparative resistance of rabbit and mouse red cells.—A 2·5 per cent suspension of mouse red cells in saline (prepared from the pooled blood of 6 animals) was added to a haemolysin dilution series in parallel with a similar series to which a rabbit erythrocyte suspension was added.

Effect of antitoxic sera.—It was desirable to test the effect on *Cl. histolyticum* haemolysin of both the antibody of an oxygen-labile haemolysin and that of an oxygen-stable haemolysin. Streptolysin O standard antitoxin (20,000 u./ml.) and Burroughs Wellcome staphylococcus antitoxin globulins (1250 u./ml.) respectively were used. The serum being investigated was added in 0·02 ml. amounts to each haemolysin dilution in an ordinary titration before the addition of the red cells. A simple titration was performed as a control.

Plate demonstration of serological relationship of oxygen-labile haemolysins.—Human red cells, washed four times, were re-suspended in normal saline to the original volume. A base medium consisting of Todd-Hewitt broth with the addition of 0·02 per cent sodium thioglycollate and 1·5 per cent New Zealand agar was used. Plates were prepared by pouring initially 8 ml. of this medium into 3½ in. (8·9 cm.) Petri dishes. When the agar had set, a sterile filter-paper strip, soaked in undiluted antistreptolysin O serum, was placed across the surface of each plate. A covering layer of 8 ml. of medium, with the addition of 0·4 ml. of the human red cell suspension, was then poured.

Cultures of pneumococcus, type II (N.C.T.C. No. 7466), *Cl. tetani* type I (U.S.A. II strain, N.C.T.C. No. 279), *Cl. welchii*, type A (S 107 CN 1491 strain, N.C.T.C. No. 8237) and *Cl. histolyticum* (N.C.T.C. No. 503) were each streaked across a plate at right angles to a strip. They were incubated anaerobically at 37° for up to 3 days. Areas of haemolysis appeared after 24 hr. incubation with *Cl. welchii*, and after 48 hr. with *Cl. tetani* and pneumococcus. *Cl. histolyticum* was non-haemolytic on these plates ; but haemolysis was induced by increasing the glucose content of the medium tenfold (from 0·2 to 2·0 per cent) and incubating for 72 hr.

The effect of the diffusion of antistreptolysin O from the strip on the production of haemolysis by each organism was observed.

RESULTS.

The findings are set out in the Table.

The addition of 0·2 M hydrogen peroxide to *Cl. histolyticum* haemolysin completely removed its lytic power, confirming the property of oxygen-lability observed by Bowen (1952).

Exposure to cholesterol at a ligroin-water interface reduced the haemolytic titre to about one-eighth of its initial value, ligroin alone having no effect. This is a similar order of inhibition to that found when streptolysin O was similarly treated (Howard *et al.*, 1953).

Mouse red cells appeared to be about four times as resistant to *Cl. histolyticum* haemolysin as those of the rabbit.

Haemolysis with all concentrations of lysin was completely abolished by the addition of high-titre antistreptolysin O serum. The minimal inhibition following addition of the staphylococcus antitoxin is attributable to the small amount of antistreptolysin O invariably present in horse serum. A similar slight inhibitory effect was produced by the addition of normal horse serum.

Plates 1-4 show the inhibition of *Cl. histolyticum* haemolysin and three other O-labile haemolysins by antistreptolysin O serum. *Cl. welchii* α toxin, producing the large area of partial (hot-cold type) haemolysis, is uninhibited, but the narrow inner zone of haemolysis due to the θ toxin is completely inhibited. When an $\alpha\delta$-haemolysin producing strain of staphylococcus was grown on a similar plate (with rabbit cells substituted for human) in 30 per cent CO_2, there was almost no inhibition of the haemolysins produced.

TABLE.—*Activity of* Cl. histolyticum *Haemolysin against Rabbit Red Cells under Different Conditions.*

	Dilution of culture supernatant.							
	2/5	1/5	1/10	1/20	1/40	1/80	1/160	1/320
Effect of oxidation.								
Reduced (0·2 M thioglycollate)	100	100	90	80	60	40	5	0
Oxidised (0·2 M hydrogen peroxide)	0	0	0	0	0	0	0	0
Inhibition by cholesterol.								
Reduced control	100	100	100	95	90	80	—	—
Previously exposed to cholesterol at ligroin-water interface	100	100	80	50	2	0	—	—
Previously exposed to ligroin only	100	100	100	100	95	90	—	—
Comparative resistance of rabbit and mouse red cells.								
Reduced, with 2·5 per cent rabbit red cell suspension	100	100	90	80	60	40	5	0
Reduced, with 2·5 per cent mouse red cell suspension	90	80	50	30	10	2	0	0
Inhibition by antistreptolysin O.								
Reduced control	100	100	100	95	90	80	—	—
Antistreptolysin O previously added	0	0	0	0	0	0	—	—
Effect of addition of staphylococcus antitoxin.								
Reduced control	100	100	90	80	60	40	5	0
Staphylococcus antitoxin previously added	100	90	80	50	20	5	0	0

The figures represent percentage haemolysis.

DISCUSSION.

Cl. histolyticum haemolysin appears to be a typical oxygen-labile haemolysin. Its neutralisation by cholesterol and by the antibody to another oxygen-labile haemolysin place it in the group of bacterial toxins typified by streptolysin O. All the other oxygen-labile haemolysins conform to a remarkably similar pattern, with the possible exception of the δ toxin of *Cl. oedematiens*, type A (Oakley, Warrack and Clarke, 1947). The relation of the latter to this group has not yet been determined.

Although the toxins of this group are invariably lethal when administered to laboratory animals in sufficiently large doses, it is unlikely that the haemolysin of *Cl. histolyticum* plays any significant part in infection caused by this organism. Bowen found that the haemolytic activity of *Cl. histolyticum* culture filtrates could be selectively adsorbed without reducing their lethality. The pathological effects of this bacterium are almost certainly due entirely to the lethal (α) toxin and the potent proteolytic enzymes (β and γ) which it produces (Oakley and Warrack, 1950.

SUMMARY.

Clostridium histolyticum haemolysin is oxygen-labile and is inhibited by cholesterol. It is antigenically related to the other oxygen-labile haemolysins, being neutralised by antistreptolysin O. It thus belongs to the well defined class of bacterial toxins composed of streptolysin O, pneumolysin, tetanolysin and *Cl. welchii* θ toxin, all of which share these characteristics.

Mouse erythrocytes are four times as resistant to *Cl. histolyticum* haemolysin as those of the rabbit. This approximates to the degree of resistance exhibited by mouse red cells to tetanolysin, but is less than the greater resistance to streptolysin O, pneumolysin and *Cl. welchii* θ toxin.

I am grateful to Dr. L. F. Hewitt for a gift of Streptolysin O Standard Antitoxin.

REFERENCES.

Bowen, H. E.—(1952) *Yale J. Biol.*, **25**, 131.
Herbert, D.—(1941) *Biochem. J.*, **35**, 1116.
Howard, J. G., Wallace, K. R., and Wright, G. Payling—(1953) *Brit. J. exp. Path.*, **34**, 174.
Idem and Wallace, K. R.—(1953) *Ibid.*, **34**, 181.
Mita, T.—(1934) *Japan J. exp. Med.*, **12**, 285.
Oakley, C. L., and Warrack, G. Harriet.—(1950) *J. gen. Microbiol.*, **4**, 365.
Iidem and Clarke, P. H.—(1947) *J. gen. Microbiol.*, **1**, 91.
Stewart, S. E.—(1936) *Pub. Hlth. Rep., Wash.*, **51**, 1272.
Todd, E. W.—(1934) *J. Path. Bact.*, **39**, 299.—(1941) *Brit. J. exp. Path.*, **22**, 172.
Van Heyningen, W. E.—(1950) "Bacterial Toxins." Oxford (Blackwell Scientific Publications).

TUBERCLE BACILLI RESISTANT TO ISONIAZID: VIRULENCE AND RESPONSE TO TREATMENT WITH ISONIAZID IN GUINEA-PIGS AND MICE.

MARGARET BARNETT, S. R. M. BUSHBY AND D. A. MITCHISON.*

From the Wellcome Research Laboratories, Beckenham, Kent, and the Department of Bacteriology, Postgraduate Medical School of London, London, W.12.

Received for publication June 11, 1953.

In an earlier publication (Barnett, Bushby and Mitchison, 1953) 3 strains of tubercle bacilli of low resistance to isoniazid were shown to be fully virulent in mice and guinea-pigs and treatment of these animals with isoniazid gave almost complete protection. The purpose of our present investigation was to determine the virulence of strains with higher degrees of resistance, and to see whether the disease produced was influenced by treatment with isoniazid.

METHODS.

Strains.—These comprised the virulent laboratory strain, H37Rv; a variant of H37Rv, B, made highly resistant to isoniazid by transfers in sub-inhibitory concentrations of isoniazid in Dubos Tween-albumin medium (Barnett *et al.*, 1953) and 3 resistant strains, 9652, 3073 and 2736, obtained from the sputa of patients under treatment with isoniazid.

Variant B was grown in Dubos medium containing 25 μg. isoniazid/ml. in Exp. 1, and 10 μg. per ml. in Exp. 2. The resulting growth was standardized by weight in a vaccine centrifuge tube and injected into mice and guinea-pigs.

The strains from patients were isolated on isoniazid-free Löwenstein slopes, and then inoculated on a series of Löwenstein slopes containing a range of isoniazid concentrations. Subcultures were made from the slope containing the highest concentration of isoniazid on which growth was of the same degree as on the control isoniazid-free slope. Subsequent cultures were maintained on slopes containing this concentration of isoniazid which was 0·2 μg./ml. for 9652, 1 μg./ml. for 3073, and 50μg/ml. for 2736. In this way any admixture of organisms which had a lower degree of resistance was eliminated. Suspensions for infecting the animals were obtained by scraping off the growth as soon as this became apparent and shaking a weighed amount for 5 minutes in bottles containing glass beads and Dubos medium. The strain H37Rv was grown either in Dubos medium or on Löwenstein slopes free of isoniazid. Before infecting the animals sensitivity tests and viable counts were set up from all the suspensions.

Sensitivity tests.

Sensitivity tests to isoniazid were carried out in Dubos medium and on Löwenstein slopes as described previously (Barnett *et al.*, 1953). The results obtained on Löwenstein slopes probably give the better measure of the degree of resistance (Mitchison, 1953). In addition a rough assessment of the proportion of the population capable of growth at various isoniazid concentrations was obtained in Exp. 4 and 5 by sowing the suspension, undiluted and diluted 1 : 100 and 1 : 10,000, with a calibrated loop on Löwenstein slopes.

Viable counts.

The number of viable units, *i.e.*, clumps of organisms developing into single colonies, was determined by the method of Fenner (1951) using oleic acid-albumin agar, containing 0·5 per cent bovine fraction V albumin but no yeast extract.

* In receipt of a grant from the Medical Research Council.

Animal experiments.

In each experiment mice and guinea-pigs were infected with the same suspension on the same day. White mice of the Schofield strain in groups of 10 were injected intravenously with 1·2 or 1·0 mg. and 0·3 mg. of bacilli (wet weight). Guinea-pigs were injected intramuscularly in the right thigh with 0·1 mg. of bacilli. The treatment with isoniazid was begun immediately after infection, and was continued until the animals either died or were killed at the end of the experiment. Except in Exp. 5, the drug was given subcutaneously and the dose was either 10 mg./kg. or 2 mg./kg., given at 9 a.m. and 5 p.m. daily, except on Saturdays, when only one dose, and on Sundays when none, was given. Guinea-pigs were weighed at fortnightly intervals and the dose adjusted accordingly.

Post-mortem examinations and in some cases histological and cultural examinations were made on the lungs of mice. Complete post-mortem examinations were made on all guinea-pigs, and a histological examination was made of the spleen, liver and lungs of all animals in which there was little or no macroscopic tuberculosis and on a sample of the remaining animals. Portions of the spleens and, in some cases, the inguinal glands of guinea-pigs were cultured on Löwenstein slopes. Where there was macroscopic tuberculosis in the spleen a range of slopes containing isoniazid was inoculated directly with the tissue suspension, but where there was no macroscopic tuberculosis sensitivity tests were done on colonies from the primary culture. Negative cultures were incubated for 8 weeks.

Mantoux tests with 0·1 ml. of dilutions of Old Tuberculin (OT) were carried out in Exp. 2, 4 and 5, and were read after 48 hours. Guinea-pigs in Exp. 2 and 4 had coloured skins and the results will be referred to briefly. Those in Exp. 5 were all white.

RESULTS

Experiments 1 *and* 2.

In these experiments H37Rv and the variant B were compared. Variant B grew in 25 but not 50 μg. isoniazid/ml. in Dubos medium after 14 days' incubation and on Löwenstein slopes, it grew on 200 but not 500 μg./ml. In the first experiment each strain was injected into 12 guinea-pigs which were divided into 3 groups receiving either no treatment or treatment at 4 or at 20 mg./kg./day. Half of each group of 4 animals was killed at 42 days and half at 63 days. In the second experiment 14 guinea-pigs, of which 4 were treated, were infected with variant B. These were killed at intervals, the last six at 144–147 days. A control group of 4 untreated animals was infected with H37Rv. The mouse tests were terminated at 81 days.

Results.

Guinea-pigs.—In Exp. 1 untreated animals infected with H37Rv developed extensive tuberculosis of a degree similar to that described and assessed in Exp. 5, and those treated (at both levels) were completely protected. The histological appearances of the organs of the treated animals were normal and cultures from their spleens were negative, as in all subsequent experiments with one exception noted in Exp. 5.

Guinea-pigs infected with variant B showed little evidence of tuberculosis. A caseous abscess was found at the site of inoculation in 3 out of 12 animals. The draining lymph glands were usually enlarged, and 6 of them contained caseous foci of variable size. There was no macroscopic evidence of tuberculosis in the spleens, livers or lungs of any of the animals except that in one, killed after 42 days, minute tubercles in the spleen and liver were just visible.

The histological and cultural findings are summarized in Table I. Sections of the livers of animals killed after 42 days contained small collections of from 10–100

TABLE I.—*Results of Histology, Spleen and Inguinal Gland Cultures and Isoniazid Resistance of Recovered Strains in Guinea-pigs.*

Strain.	Inhibitory concentration of isoniazid (μg./ml.).*	Dose of isoniazid (mg./kg./day).	Experiment.	Period of infection.	Histology of spleen, liver and lungs.†	Result of spleen and inguinal gland cultures: No. positive/No. cultivated. Spleen.	Inguinal gland.	Degree positive.‡	Inhibitory concentration of isoniazid (μg./ml.).*
H37Rv	0·2	0	1, 2, 4, 5	42–107	+++	7/7	—	+++ to +	0·2(7)
H37Rv	0·2	4, 20	1, 4, 5	42–77	0	1/14	—	4	200
B	500	0, 4, 20	1, 2	42	±	4/6	—	1 to 5	50 (4)
B	500	0, 4, 20	1, 2	63–146	± to 0	0/18	—		
9652	5	0, 20	4, 5	42–77	+++	11/12	—	+++ to 1	5 (5), 10 (5), 50 (1)
3073	50	0, 20	4	42	++ to ±	4/4	—	++, ++, 9, 6	50 (4)
3073	50	0. 20	4, 5	71, 77	++ to 0	3/8		++, 7, 6	10 (1), 50 (2)
							4/4	+++ to +	50 (3), 200 (1)
2736	200	0, 20	4	42	± to ±	2/4		2, 4	200 (2)
							2/2	++	200 (2)
2736	200	0, 20	4, 5	71, 77	0	0/16			
							3/6	1 to 3	200 (2), 500 (1)

* 28-day reading on Löwenstein medium. The figures in brackets indicate the number of isolations.

† +++ = Extensive tuberculosis.
++ = Less extensive tuberculosis.
\+ = Small tubercles.
± = Collections of 10–100 histiocytes.
± = Collections of up to 10 histiocytes.
0 = Normal histology.

‡ = +++ to + degrees of growth. Numbers indicate the numbers of colonies.

histiocytes infiltrated with lymphocytes. Similar but less numerous lesions were found in the spleen and lungs. No acid-fast bacilli were found in these lesions, but spleen cultures from 2 out of the 6 guinea-pigs were positive, yielding scanty colonies of the same degree of isoniazid resistance as the suspension used for infecting the animals. On the other hand in animals killed after 63 days the organs were histologically normal, or contained lesions composed of up to 20 histiocytes which appeared to be regressing. The 6 spleen cultures were negative.

The organisms isolated from 2 of the spleens of animals infected with variant B were injected into 4 guinea-pigs. At 138 days later there was no macroscopic or histological evidence of tuberculosis except in one where there was a caseous abscess at the site of inoculation and a caseous inguinal gland, from which cultures failed to yield tubercle bacilli.

The regressive nature of the lesions produced by the variant B was clearly shown in Exp. 2, where the period of observation was extended. In both of two guinea-pigs killed at 42 days there were small areas of caseation in the inguinal glands. Similar lesions were found in 4 out of 6 animals killed at 70 or 98 days. However, the only evidence of tuberculosis in 6 animals killed at 144–147 days was a small abscess at the site of inoculation in one.

The organs of guinea-pigs killed at 42 days contained microscopic lesions similar to those of animals in Exp. 1 killed after the same period, and cultures of both spleens yielded scanty colonies of resistant bacilli. On the other hand the histological appearance of the organs was normal and no tubercle bacilli were isolated from the spleens of animals killed after 70 days or more.

In neither experiment did treatment with isoniazid influence any of the findings in animals infected with variant B.

The animals infected with H37Rv in the second experiment developed the usual extensive tuberculosis; two were killed after 42 days and two died after 94 and 107 days.

Mice.—The protocol of the mouse test of Exp. 1 is given in Table II. Treatment with isoniazid protected the mice infected with H37Rv except for 3 animals which died after 51, 54 and 66 days with macroscopic tubercles in their lungs. Variant B was distinctly less virulent than H37Rv, though still capable of killing 70 per cent of untreated mice infected with 1·2 mg. Treatment afforded no protection, and, in fact, it appeared to decrease the number of survivors and the survival time.

In Exp. 2 the mice were treated at the one-dose level of 20 mg./kg. and the results are included in Table II. In this test the variant B was incapable of causing fatal tuberculosis in untreated mice, but 8 out of the 10 treated animals died with gross macroscopic tuberculosis. Histological examination of the lungs of the untreated surviving animals showed only small patches of infiltration with histiocytes and lymphocytes in which tubercle bacilli were scanty or absent, and similar lesions but somewhat more marked were present in the treated survivors. The surviving treated mice infected with H37Rv showed a variable degree of histological tubercle formation in which no acid-fast bacilli were found.

Experiment 3.

In this experiment four guinea-pigs were infected with variant B after 10 subcultures in Dubos medium free of isoniazid, during which it retained its resistance. The animals received no treatment and were killed after 85 days. This

TABLE II.—*Chemotherapeutic Experiment in Mice with H37Rv and its Resistant Variant B.*

Experiment.	Strain.	Infecting dose per mouse: mg.	Infecting dose per mouse: Viable units.	Dose of isoniazid (mg./kg./day).	Number of mice out of 10 surviving on day: 15.	30.	42.	66.	81.	Average day survival at 81 days.	Appearance of lungs of survivors.
1	H37Rv	1·2	$5·6\times10^7$	—	2	0	0	0	0	10·7	—
		1·2		4	10	10	10	9	9	77·9	+
		1·2		20	10	10	10	10	10	81·0	±
		0·3	$1·4\times10^7$	—	6	1	0	0	0	18·6	—
		0·3		4	10	10	10	9	9	78·1	±
		0·3		20	10	10	10	9	9	79·4	±
	B	1·2	$2·9\times10^7$	—	7	5	3	3	3	37·3	±
		1·2		4	7	5	1	1	0	28·6	—
		1·2		20	5	2	2	0	0	22·5	—
		0·3	$7·1\times10^6$	—	10	10	10	10	9	80·0	±
		0·3		4	10	9	9	9	9	75·4	±
		0·3		20	10	9	8	5	3	57·1	±
2	H37Rv	1·0	$7·2\times10^7$	—	0	0	0	0	0	9·1	—
		1·0		20	10	10	10	10	10	81·0	±
		0·3	$2·2\times10^7$	—	10	6	3	0	0	33·7	—
	B	1·0	*	—	9†	8†	8	8	8	67·7	±
		1·0		20	9	6	4	3	2	44·9	+
		0·3	*	—	10	10	10	10	10	81·0	±

* No growth on oleic acid-albumin agar plates.
† 2 mice died from non-tuberculous causes.
± = more distended than normal.
+ = few small tubercles.

organism was still non-invasive, for the only evidence of tuberculosis was caseous abscesses at the site of inoculation in three of the animals.

Experiments 4 and 5.

In these experiments H37Rv and the three isoniazid-resistant strains from patients were compared. These strains had been subcultivated twice on isoniazid-containing medium and the degrees of resistance are shown in Fig. 1. It can be seen that in Exp. 4 the majority of the population of 9652 was inhibited by 5 μg./ml. Strain 3073 was slightly more resistant, most of the organisms being inhibited by 10 μg./ml., and strain 2736 was highly resistant in a degree comparable to variant B. For Exp. 5 the strains had been subcultivated four times on isoniazid containing medium, and the degree of resistance remained unaltered except that the small component of strain 9652 capable of growth at 5 μg./ml was absent.

In Exp. 4 each of the 4 strains was injected into 8 guinea-pigs, of which 4 were untreated and 4 were treated with isoniazid at 20 mg./kg./day. Half of these animals were killed after 42 days and the remainder after 77 days. One treated guinea-pig infected with 9652 died at 74 days. The mouse test was terminated at 83 days.

In Exp. 5 the strains were injected into larger groups of animals. Strains H37Rv, 3073 and 2736 were each injected into 12 guinea-pigs, and 9652 was injected into 18 guinea-pigs. Half of these animals were untreated, and the other half were treated with isoniazid at 20 mg./kg./day. One treated animal infected with 9652 and one untreated animal infected with H37Rv died from

non-tuberculous causes on the 25th and 40th days respectively. The remaining animals were killed at 71 days. The mouse test was terminated at 100 days.

Results.

Guinea-pigs.—The results of Exp. 4 are shown in Fig. 2. Table V gives a comparison of the degrees of tuberculosis produced by each strain in Exp. 4 and 5 assessed as follows: Out of a total of 100 points a maximum of 32 each was allocated to the spleen and liver, 20 to the lungs and 16 to the local abscess and lymph

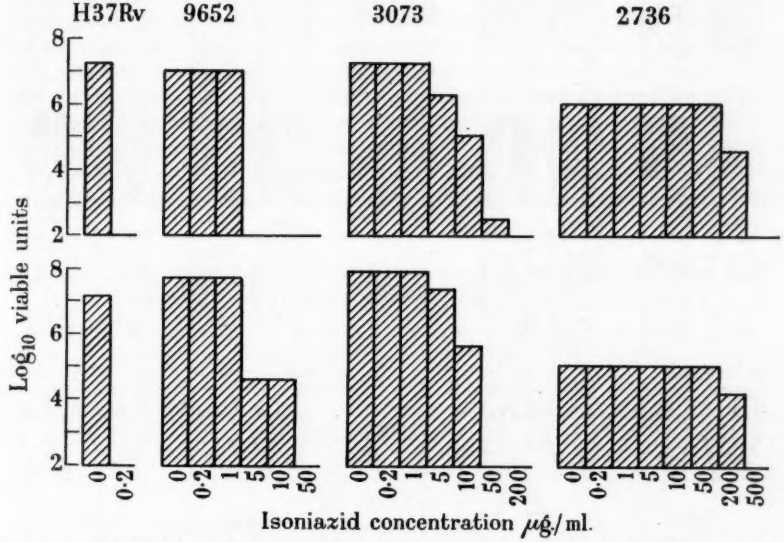


FIG. 1.—The isoniazid sensitivity of H37Rv and 3 resistant strains from patients. Approximate numbers of viable units per mg. of the strains capable of growth on Löwenstein slopes containing a range of isoniazid concentrations. Results read after 42 days' incubation which did not differ significantly from those after 28 days.

Top line—Exp. 5.
Bottom line—Exp. 4.

glands. A maximum of 5 points was allocated to histological involvement of a macroscopically normal spleen or liver and 3 to a similar lung. The histological and cultural results of both experiments are summarised in Table I.

In Exp. 4 untreated animals infected with H37Rv developed a lesser degree of tuberculosis than was usual, but, in Experiment 5, the strain of H37Rv was again fully virulent.

Strain 9652, having the lowest degree of resistance, caused extensive tuberculosis. In Exp. 5, after exclusion of non-tuberculous deaths, the amount of disease found in treated guinea-pigs infected with this strain was not significantly different from that found in untreated animals. The amounts of tuberculosis in untreated animals infected with H37Rv and in untreated animals infected with 9652 were also not significantly different but if comparison is made between the untreated

H37Rv group and both treated and untreated animals infected with 9652 the former had a slightly larger amount of tuberculosis. ($P = 0{\cdot}02 - 0{\cdot}01$). One can therefore conclude that 9652 was probably slightly less virulent than a fully virulent strain of H37Rv.

Strain 3073, with an intermediate degree of resistance, appeared to be of low virulence, producing a small but variable amount of tuberculosis. There was a

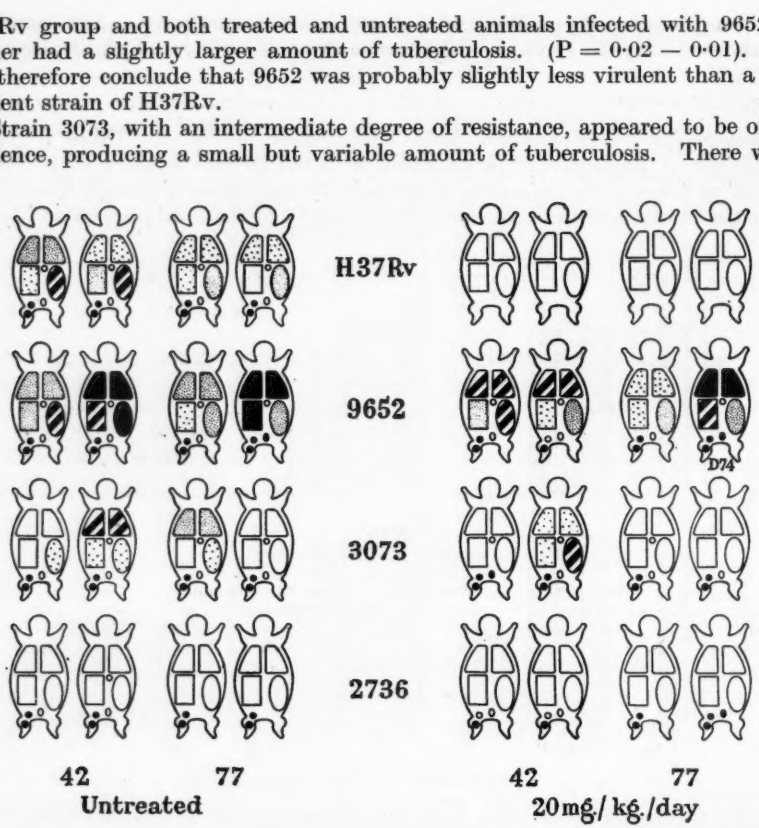


FIG. 2.—Chemotherapeutic experiment in guinea-pigs with H37Rv and 3 resistant strains from patients. Result of Exp. 4. The extent of tuberculosis in the organs is indicated by the degree of shading, complete shading indicating maximal involvement and stippling minimal involvement. The liver is indicated by a rectangle and the spleen by an oval. From the right leg upwards are shown the site of inoculation, the draining inguinal glands, the sublumbar gland and the coeliac gland. Enlarged glands are marked by open circles, which are shaded if there was any degree of caseation visible macroscopically.

suggestion that the tuberculosis caused by this strain healed slightly during the period between the 42nd and 77th day (see Fig. 2). Thus in the animals killed at 42 days, 3 had macroscopic disease in the lungs, liver or spleen and the 4th had microscopic lesions. In the 4 killed at 77 days 1 had macroscopic disease in these organs but they were entirely normal in the other three.

Strain 2736, with a high degree of resistance, behaved in a manner very similar to that shown by B in Exp. 1 and 2, indicating that it had lost the ability to produce progressive tuberculosis. Once more histological lesions and spleen cultures became negative after the 42nd day (see Table I). In Exp. 4 cultures of two inguinal glands from animals killed at 42 days yielded heavy growths, whereas, in

Exp. 5, only 3 out of 6 such cultures from animals killed at 71 days grew a few colonies. Thus organisms in the inguinal glands also appeared to die out between the 42nd and 71st days.

Treatment with isoniazid completely protected guinea-pigs infected with H37Rv. Unexpectedly one of their spleen cultures yielded 4 colonies of highly resistant bacilli. Both the macroscopic and histological appearance of the organs of this animal were normal. The strain was injected into two guinea-pigs, which were killed at 42 days, during which they received no treatemnt. They both had abscesses at the site of inoculation and caseous tubercles in the inguinal glands, but no further evidence of tuberculosis.

Among animals infected with the three resistant strains isoniazid treatment had no influence on the macroscopic appearance at post mortem, the histological findings, the results of spleen or inguinal cultures or the degree of resistance found in recovered strains. Sensitivity tests on cultures recovered from the spleens and inguinal glands showed no alteration in resistance.

Mice.—The protocol of Exp. 4 is given in Table III. The low degree of virulence of the H37Rv strain, apparent in the guinea-pigs, was also found in the mice since, even with the 1·0 mg. dose 40 per cent of the untreated mice survived.

TABLE III.—*Chemotherapeutic Experiment in Mice with H37Rv and 3 Resistant Strains from Patients. Result of Experiment* 4.

Strain.	Infecting dose per mouse. mg.	Infecting dose per mouse. Viable units.	Dose of isoniazid (mg./kg./day).	Number of mice surviving out of 10 on day 12.	24†.	42.	63.	83.	Average day survival at 83 days.	Appearance of lungs of survivors.
H37Rv	1·0	$9·8\times10^6$	—	10	9	7	5	4	60·9	+++
	1·0		20	10	7	7	7	7	63·9	±
	0·3	$2·9\times10^6$	—	10	10	8	8	5	67·7	+++
	0·3		20	10	7	7	7	7	64·2	±
9652	1·0	$2·2\times10^7$	—	10	3	2	0	0	26·8	—
	1·0		20	9	4	2	0	0	22·6	—
	0·3	$6·5\times10^6$	—	10	8	7	3	2	52·7	+++
	0·3		20	9	2	1	0	0	21·7	—
3073	1·0	$4·3\times10^7$	—	10	10	10	10	9	82·6	++
	1·0		20	8	2	0	0	0	18·9	—
	0·3	$1·3\times10^7$	—	10	10	10	10	10	83·0	++
	0·3		20	9	6	4	3	0	37·3	—
2736	1·0	*	—	10	10	10	10	10	83·0	+++
	1·0		20	9	3	1	0	0	22·0	—
	0·3	*	—	10	10	10	10	10	81·4	++
	0·3		20	9	6	5	5	5	51·8	++

* No growth on oleic acid-albumin agar plates.
† Up to the 24th day some of the deaths were attributable to non-tuberculous causes.
± = more distended than normal.
\+ = few small tubercles.
++ = few large tubercles.
+++ = many large tubercles.

Unfortunately, up to the 24th day there were 3 (out of 10) non-tuberculous deaths in this experiment in both the treated groups of mice infected with H37Rv and 8 in various other groups. However, the mice that died from about the 24th day onwards showed typical macroscopic tuberculosis at post mortem and no further deaths occurred in the treated H37Rv groups.

In spite of this defect one can draw the following conclusions: Strain 9652 was virulent in mice, though the degree of virulence could not be assessed. Treatment with isoniazid certainly did not markedly protect the mice infected with this strain, but whether the survival rate was altered is not known, since the apparent decrease might have been due to the non-tuberculous deaths. Strains 3073 and 2736, though capable of causing tubercle formation, were not able to kill untreated animals, except for 2 mice dying on the 67th and 80th days. There is evidence that isoniazid treatment increased the virulence of these strains, since a number of the treated animals died with typical tuberculosis in the later period of the experiment when no adventitious deaths were occuring, and the increase was most marked in the animals infected with the higher dose.

In Exp. 5 the intermediate resistant strain 3073 was not used and the treated animals infected with H37Rv, 9652 and 2736 were given the drugs at dose levels 20, 4 and 0·8 mg./kg./day by the subcutaneous route. Groups were also included in which treatment at 20 mg./kg. was given in the diet to exclude the possiblity that deaths had been accelerated by the procedure of subcutaneous injection.

The results are shown in Table IV. Isoniazid at the 20 mg./kg. level given

TABLE IV.—*Chemotherapeutic Experiment in Mice with H37Rv and 2 Resistant Strains from Patients. Result of Experiment 5.*

Strain.	Infecting dose per mouse: mg.	Infecting dose per mouse: Viable units.	Dose of isoniazid (mg./kg./day).	Route.	Number of mice surviving out of 10 on day: 20.	40.	60.	80.	10.	Average day survival at 100 days.	Appearance of lungs of survivors.
H37Rv	1·0	$2·8\times10^7$	—	—	0	0	0	0	0	6·9	—
	1·0		20	S.C.	10	10	10	10	10	100·0	±
	1·0		4	S.C.	10	10	10	10	9	98·9	±
	1·0		0·8	S.C.	8	3	1	0	0	31·0	—
	1·0		20	Diet	3	3	3	3	3	32·9	—
	0·3	$8·4\times10^6$	—	—	7	1	1	0	0	26·7	—
	0·3		20	S.C.	10	10	10	10	10	100·0	±
	0·3		4	S.C.	10	10	10	10	10	100·0	±
	0·3		0·8	S.C.	10	10	10	9	8	95·1	+
	0·3		20	Diet	10	10	10	9	9	97·2	±
9652	1·0	$3·2\times10^7$	—	—	5	0	0	0	0	21·6	—
	1·0		20	S.C.	5	0	0	0	0	24·0	—
	1·0		4	S.C.	0	0	0	0	0	16·9	—
	1·0		20	Diet	3	0	0	0	0	19·8	—
	0·3	$9·6\times10^6$	—	—	6	1	0	0	0	24·6	—
	0·3		20	S.C.	7	3	2	0	0	34·8	—
	0·3		4	S.C.	5	2	1	0	0	29·7	—
	0·3		20	Diet	7	0	0	0	0	25·8	—
2736	1·0	*	—	—	9	8	6	6	5	71·8	++
	1·0		20	S.C.	10	10	9	8	6	90·2	+
	1·0		4	S.C.	10	10	10	9	5	92·1	+
	1·0		0·8	S.C.	10	10	9	6	6	86·0	+
	1·0		20	Diet	10	10	9	5	3	81·1	±
	0·3	*	—	—	10	10	10	9	8	96·5	++
	0·3		20	S.C.	10	9	8	7	6	83·0	+
	0·3		4	S.C.	10	10	10	10	10	100·0	+
	0·3		0·8	S.C.	10	10	10	10	10	100·0	++
	0·3		20	Diet	10	10	10	10	8	97·1	+

* No growth on oleic acid-albumin plates.
± = more distended than normal.
\+ = few small tubercles.
++ = few large tubercles.

subcutaneously completely protected animals infected with H37Rv. When this dose of isoniazid was given in the diet 7 out of the 10 mice infected with 1·0 mg. died during the first 8 days probably because the intake of food and therefore of isoniazid was low during this period. Protection was almost complete when the dose, given subcutaneously, was 4 mg./kg. and was partial at 0·8 mg./kg. At the end of the experiment cultures were taken from the lungs of 21 of the treated mice infected with H37Rv. Of these 7 were positive and 4 were resistant to isoniazid (growth on 0·2 μg./ml.). Of the 3 sensitive strains 2 were from mice treated with the 0·8 mg. dose of isoniazid.

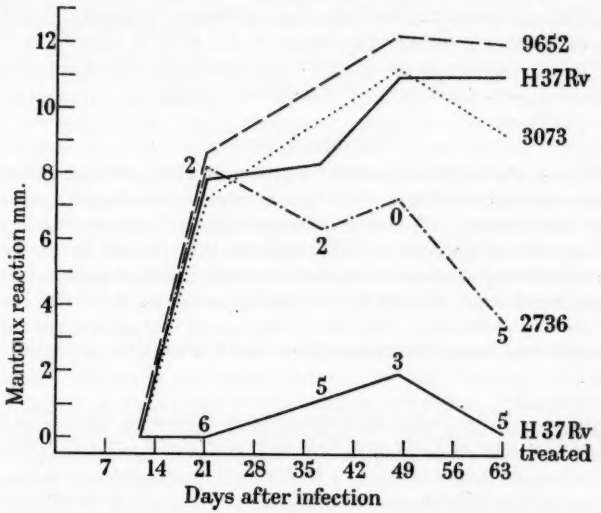


FIG. 3.—Development of allergy in guinea-pigs infected with H37Rv and 3 resistant strains from patients.

The average diameter of the Mantoux reaction to 0·1 ml. of 1 : 1000 OT is shown at intervals after infection. The figures indicate the number of animals with negative reactions (included in the average) out of 12 infected with 2736 and 6 with H37Rv (untreated).

Strain 9652 proved to be almost as virulent as H37Rv, and strain 2736 of very low virulence. The apparent increase of virulence by treatment with isoniazid was less marked than in Exp. 4, for only in the group infected with 1·0 mg. of 9652 and treated with 4 mg./kg. was there a significantly shorter survival than in the untreated controls ($P = 0·02 - 0·01$). No enhancing effect was seen in the corresponding group treated with 20 mg./kg., but since 9652 had a low degree of resistance the higher treatment level might have partially inhibited multiplication in the mice. The fact that there was no apparent increase in virulence with the higher dose of isoniazid excludes the possibility of the effect being due to toxicity.

Mantoux reactions.

In Exp. 5 serial Mantoux reactions were done with 1 : 1000 OT and the results are shown in Fig. 3. The reactions in the treated guinea-pigs infected with H37Rv

remained negative except for 5 transitory weak reactions in 4 of the animals. This was also observed in similar animals in Exp. 4. In all the remaining groups allergy developed to the same degree between the 12th and 21st day. After the 21st day the degree of allergy in animals infected with 2736 decreased gradually, whereas in those infected with 9652 and H37Rv (untreated) it increased. A similar decline in allergy may have begun after the 49th day in those infected with 3073. Retesting with 1 : 10 OT after 65 days indicated a low level of allergy in guinea-pigs infected with 2736 (average diameter of reactions 13·9 mm. ± 0·77), and an even lower level in treated animals infected with H37Rv (average 8·9 mm. ± 1·2). The degree of allergy also gradually declined in guinea-pigs infected with variant B in Exp. 2 between the 35th day (average 13·9 mm. ± 0·9 with 1 : 100 OT) and the 134th day (average 10·2 mm. ± 2·2 with 1 : 10 OT). On the 143rd day the surviving animals were injected intraperitoneally with 1 ml. undiluted OT and 5 out of 6 died with signs of tuberculin shock.

Viability of strains.

In both Exp. 4 and 5 the suspensions of strain 2736 prepared from 10- and 21-day Löwenstein cultures did not grow on the oleic acid-albumin agar plates, even after 6 weeks' incubation. One could conclude that there were at least 1000 times fewer bacilli capable of growth on this medium than would be expected from the counts on the remaining strains which were normal. Subsequent experiments with this strain indicated that a small but variable proportion of the inoculum could grow under these conditions. On the Löwenstein slopes growth occurred, but the viable count was about 100 times lower than would be expected.

TABLE V.—*The Degree of Tuberculosis Found in Guinea-pigs Infected with H37Rv and 3 Resistant Strains from Patients.*

Strain.	Inhibitory concentration of isoniazid (μg./ml.).	Dose of isoniazid (mg./kg./day).	Average degree of tuberculosis in experiment number. 4.	5.
H37Rv	0·2	—	47·5 (4)	75·4 (5)
		20	0 (4)	0 (6)
9652	5	—	70·0 (4)	63·1 (9)
		20	57·7 (4)	54·7 (8)
3073	50	—	30·7 (4)	24·7 (6)
		20	23·7 (4)	23·0 (6)
2736	200	—	14·5 (4)	9·5 (6)
		20	12·0 (4)	11·7 (6)

The degree of tuberculosis was assessed out of a possible total of 100 as explained in the text. Animals dying from non-tuberculous causes in Exp. 5 were excluded. The figures in brackets are the numbers of animals on which each average is based.

In Exp. 1 the viable count on the suspension of variant B was normal. However in Exp. 2 there was no growth on the plates and, in spite of contamination of many of the plates, one could also conclude that there were at least 100 times fewer colonies on them than would be expected from the parallel count on H37Rv. A loopful of this suspension yielded confluent growth on Löwenstein slopes. In further counts on variant B there have been irregular failures to yield the expected number of colonies on oleic acid-albumin agar plates, although growth on Löwenstein medium occurred to the expected extent.

DISCUSSION.

The main conclusion to be drawn is that there is apparently a relation between the degree of isoniazid resistance of strains of tubercle bacilli and their virulence in guinea-pigs and mice. The highly resistant strains, B and 2736, produced only minimal lesions in guinea-pigs. In mice they caused tubercle formation in the lungs which, was either incapable of killing the animals or only killed them after a longer time than was taken by the same weight of sensitive bacilli. Furthermore, the highly resistant strain isolated, in Exp. 5, from the spleen of a guinea-pig infected with H37Rv and treated with isoniazid was found to be of low virulence, although the circumstances in which resistance developed would appear to favour the selection of virulent organisms. Strain 3073, with a slightly lower degree of resistance, caused rather more extensive lesions in guinea-pigs, and these tended to regress slighly with the passage of time. In mice this strain showed the same lack of virulence as did the highly resistant strains. Strain 9652, with a lower degree of resistance, was only slightly less virulent than H37Rv in guinea-pigs and in mice. Finally, as we have previously reported (Barnett *et al.*, 1953), 3 strains with an even lower degree of resistance were fully virulent in guinea-pigs and mice.

The virulence of resistant strains appears to be a fairly stable character. Thus strain B produced the same minimal lesions following passage through guinea-pigs or after 10 subcultures in isoniazid-free Dubos medium. The virulence of strains 2736, 3073 and 9652 was also similar in Exp. 4 and 5, between which the strains were subcultivated twice.

Frequent subcultivation of H37Rv in Dubos medium may sometimes lead to a loss of virulence. Strain B was subcultivated in this medium, and this might be considered a contributory factor in the loss of virulence. However, in Exp. 1 the H37Rv control strains had been subcultivated with it in parallel and was virulent. Furthermore, in Exp. 4, H37Rv appeared to have become partially attenuated during subculture and now had about the same degree of virulence in mice as had variant B in Exp. 1. However, in guinea-pigs the attenuated H37Rv strain was still capable of causing fairly extensive tuberculosis, whereas variant B only produced minimal lesions. Thus the relatively higher virulence in mice than in guinea-pigs appears to establish that the lack of virulence of B was qualitatively different from that sometimes occurring in strains of H37Rv during subculture.

Isoniazid has a rapid bactericidal action (Mackaness and Smith, 1953). The complete lack of tuberculous lesions and the low level of allergy found in treated guinea-pigs infected with H37Rv indicate that the bacilli failed to multiply to any extent in these animals. On the other hand the local lesions, microscopic tubercles in the organs and the initial rise of allergy in animals infected with B and 2736 suggests that these highly resistant strains were capable of initial multiplication in the host. After this initial period of multiplication the bacilli tended to die spontaneously, as shown by the failure to obtain positive spleen cultures after 6 weeks (Table I), the fall in positivity of cultures from inguinal glands after 6 weeks (Table I), the tendency for lesions at the site of inoculation and in the draining glands to heal (Exp. 2) and the gradual fall in the degree of allergy after 3 weeks (Fig. 3). It is also likely that there was a period of preliminary multiplication in mice, since macroscopic tubercle formation was found in survivors injected with 2736 in Exp. 4 and 5, but not in treated mice infected with H37Rv. Since the infecting dose of bacilli was much larger on a weight-for-weight basis in mice

than in guinea-pigs, this initial growth might be responsible for the greater virulence of resistant strains to mice than to guinea-pigs.

Such a phase of intial multiplication followed by apparent death of the organisms *in vivo* is very similar to the behaviour of strain B *in vitro*, since we have shown previously (Barnett, *et al.*, 1953) that, during the first subcultures of this strain in Dubos medium containing isoniazid, fully grown cultures often failed to grow or only grew after a considerable delay when subcultivated in the same medium. Furthermore the failure to obtain growth from young cultures of variant B and of 2736 in the viable counts of the present investigation suggests that these strains had poor viability. This poor viability seems related to the medium employed, since the viable counts on Löwenstein medium in Exp. 4 and 5 were not as low as those on oleic acid-albumin agar.

The lack of virulence in mice of strain 2736 in Exp. 4 and 5 and of variant B in Exp. 2 might be due completely or in part to the death of a proportion of the organisms in the infecting dose as shown by the low viable counts. In favour of this view is the finding in Exp. 1 that 1·2 mg. of a suspension of variant B which yielded a normal viable count killed all the untreated mice after an average of 32·9 days whereas, in experiment 2, 1·0 mg. of a suspension of this strain, which did not grow in the viable count, failed to kill any of the untreated mice. However, in Exp. 1 variant B was considerably less virulent than H37Rv, and in Exp. 4, strain 3073 was as virulent as 2736, although in both cases the counts on variant B and 3073 were normal. Thus the low viable counts on the suspensions of these resistant strains can explain only in part their lack of virulence.

The apparent increase of virulence of resistant strain in mice which are being treated with isoniazid should be treated with reserve. Although this was observed in Exp. 1, 2 and 4 the failure to obtain a clear-cut result in Exp. 5 shows that further work is necessary to establish the effect. No such stimulation of virulence was observed in guinea-pigs, nor was there any evidence of *in vitro* dependency in any of the strains.

We have shown previously (Barnett, *et al.*, 1953) that mice and guinea-pigs infected with slightly resistant strains capable of good growth on Löwenstein slopes containing 0·2 μg./ml. but yielding sparse colonies on 1 μg./ml. were protected to a considerable extent by treatment with isoniazid at 20 mg./kg./day. Using the same dose of isoniazid the degree of protection obtained in infections caused by 9652 was very slight or absent. Thus strains that were largely inhibited by 1 μg./ml. on Löwenstein slopes produced infections which responded *in vivo* to treatment with isoniazid, whereas those capable of good growth on 1 μg./ml. produced infections which did not respond to treatment but were found to have lost part or all or their virulence.

Nevertheless patients in the Medical Research Council (1952) trials who developed resistant strains (growth on Löwenstein slopes containing 1 μg./ml. or more) benefited less from treatment with isoniazid than did those whose strains remained sensitive. This appears to imply that resistance strains were to some extent virulent in man. However, it remains possible that the treatment with isoniazid may have increased the virulence of the strains as it appeared to have done in the mice in Exp. 1, 2 and 4. Furthermore highly resistant strains from patients may contain bacilli with a lower degree of resistance, and the resistance of strains in the sputum may not be the same as the resistance of bacilli in areas of the lungs containing progressive tuberculous lesions. Thus it is possible that extension of

tuberculosis in patients with resistant strains in the sputum may be caused by organisms with a low degree of resistance and a relatively high degree of virulence, similar in behaviour to strain 9652.

SUMMARY.

A highly isoniazid-resistant variant of the H37Rv strain of *Myco. tuberculosis* and a similarly resistant strain from a patient, both capable of growth on Löwenstein slopes containing 50 μg./ml. isoniazid, produced minimal tuberculous lesions in untreated guinea-pigs, which tended to heal spontaneously, and had a low degree of virulence when injected intravenously in mice. In guinea-pigs there was evidence from resolution of the tuberculous lesions, cultures of the spleens and inguinal glands and serial Mantoux reactions that the bacilli multiplied initially in the host and then died out. The low virulence found in these strains differed from that occasionally found in the H37Rv strain after numerous subcultures in Dubos medium, since there was a relatively greater lack of virulence in guinea-pigs than in mice.

The results of similar experiments with 2 strains of a lower degree of resistance suggested a negative correlation between virulence and degree of isoniazid resistance. The degree of virulence of resistant strains was not altered by subculture or by passage through guinea-pigs.

Treatment with isoniazid did not alter the extent of the tuberculous lesions in any of the guinea-pigs infected with these strains. However, in three experiments the survival period of mice infected with resistant strains was decreased by treatment with isoniazid to a greater extent at treatment levels of 20 mg. than at 4 mg./kg./day. In a fourth experiment treatment did not alter the survival period.

We are indebted to Dr. C. V. Harrison for assistance in histological interpretation and to Miss M. Monk for technical assistance. The isoniazid employed for the *in vitro* work and the guinea-pig experiments was supplied as "Nydrazid" by Messrs. E. R. Squibb, London.

REFERENCES.

Barnett, M., Bushby, S. R. M., and Mitchison, D. A.—(1953) *Lancet*, i, 314.
Fenner, F.—(1951) *Amer. Rev. Tuberc.*, **64**, 353.
Mackaness, G. B., and Smith, N.—(1953) *Ibid.*, **67**, 322.
Medical Research Council—(1952) *Brit. med. J.*, ii, 735.
Mitchison, D. A.—(1953) *J. clin. Path.*, **6**, 118.

THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY

VOL. XXXIV, NO. 5. OCTOBER, 1953.

CONTENTS

PAGE

SMITH, H., KEPPIE, J., AND STANLEY, J. L.—A METHOD FOR COLLECTING BACTERIA AND THEIR PRODUCTS FROM INFECTIONS IN EXPERIMENTAL ANIMALS WITH SPECIAL REFERENCE TO *Bacillus anthracis* . . . 471

SMITH, H., KEPPIE, J., AND STANLEY, J. L.—THE CHEMICAL BASIS OF THE VIRULENCE OF *Bacillus anthracis*. I: PROPERTIES OF BACTERIA GROWN *in vivo* AND PREPARATION OF EXTRACTS 477

KEPPIE, J., SMITH, H., AND HARRIS-SMITH, PATRICIA W.—THE CHEMICAL BASIS OF THE VIRULENCE OF *Bacillus anthracis*. II: SOME BIOLOGICAL PROPERTIES OF BACTERIAL PRODUCTS 486

BOWDEN, J.—THE ACTION OF AUREOMYCIN IN PNEUMOCOCCAL INFECTIONS IN NORMAL AND LEUCOPENIC RABBITS AND IN MICE 497

RAY, B. GHOSH, AND SWAIN, R. H. A.—SERUM ANTIBODIES IN MUMPS . 501

SMITH, WILSON, COHEN H., BELYAVIN, G., AND WESTWOOD, J. C. N.—THE EFFECTS OF ETHYL ETHER ON SOME BIOLOGICAL PROPERTIES OF INFLUENZA VIRUS 512

COOMBS, R. R. A., HOWARD, A. N., AND MYNORS, L. S.—A SEROLOGICAL PROCEDURE THEORETICALLY CAPABLE OF DETECTING INCOMPLETE OR NON-PRECIPITATING ANTIBODIES TO SOLUBLE PROTEIN ANTIGENS . 525

LOVELL, R. R. H., SCOTT, G. B. D., HUDSON, B., AND OSBORNE, J. A.—THE EFFECTS OF CORTISONE AND ADRENOCORTICOTROPHIC HORMONE ON DISPERSION OF BRUISES IN THE SKIN 535

CLARK, E. C., LATHE, G. H., AND RUTHVEN, C. R. J.—THE FRACTIONATION OF LIPIDS WHICH INHIBIT HAEMAGGLUTINATION 542

ANDERSON, K.—THE EFFECT OF STAPHYLOCOCCAL FILTRATES ON ISOLATED RABBIT SMALL INTESTINE, WITH SPECIAL REFERENCE TO ENTEROTOXIC STRAINS OF *Staph. pyogenes* 548

CHACKO, C. W.—A STUDY OF THE TREPONEMA IMMOBILIZING ANTIBODY IN SYPHILIS 556

HOWARD, J. G.—THE HAEMOLYSIN OF *Clostridium histolyticum* 564

BARNETT, MARGARET, BUSHBY, S. R. M., AND MITCHISON, D. A.—TUBERCLE BACILLI RESISTANT TO ISONIAZID: VIRULENCE AND RESPONSE TO TREATMENT WITH ISONIAZID IN GUINEA PIGS AND MICE 568

SURVEY OF PAPERS i

PRINTED BY ADLARD AND SON, LIMITED, BARTHOLOMEW PRESS, DORKING.